AUTOPHAGY

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CANCER, OTHER PATHOLOGIES, INFLAMMATION, IMMUNITY, INFECTION, AND AGING

VOLUME 4

Edited by

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Dedication

To:

Julio A. Aguirre-Ghiso, Patrice Codogno, Eduardo Couve, Ana Maria Cuervo, Guido R. Y. De Meyer, Vojo Deretic, Fred J. Dice, William A. Dunn Jr, Eeva-Lisa Eskelinen, Sharon Gorski, Tomotake Kanki, Daniel J. Klionsky, Guido Kroemer, Beth Levine, Noboru Mizushima, Yoshinori Ohsumi, Brinda Ravikumar, David Rubinsztein, Isei Tanida, Sharon A. Tooze, Herbert W. Virgin, Eileen White, Tamotsu Yoshimori, and others:

The men and women involved in the odyssey of deciphering the molecular mechanisms underlying the complexity of the autophagy process that governs our lives.

Mitophagy and Biogenesis

mTOR and nutrient sensors control
Autophagy processes in all of our cells
Dozens of proteins must play each their role
To enable engulfment of bad organelles.

Those who are young may mistakenly think one
Is safe and immune to the dangers of aging
But if you are lacking in proper PINK1
Mitochondrial fires are already raging.

For insight and knowledge some turn to the fly;
Drosophila*'s genes can help us discover*
The causes of aggregates seen in the eye,
And even find drugs to help us recover.

Ubiquitin's role in degeneration
Is to set out red flags on relevant cargo
Marking the junk that needs degradation
At a pace that is presto rather than largo.

Mitochondria fear Parkin known as PARK2
Whose ubiquitin tags on two mitofusins
Determine the fate of one or a slew,
For a lonely short life of network exclusion.

Their fate is ensured by sequestosome 1
Who recruits membranes rich with LC3-II
Autophagosome to lysosome a perfect home run
Cellular housekeeping momentarily through.

But the work isn't over and the job isn't done
Unless Paris is tagged with ubiquitin too
Then repression is lifted from PGC1
So biogenesis starts and mitos renew!

Roberta A. Gottlieb

Life in the Balance, Longevity the Goal
Self-eating, recycling, cash-for-your clunkers:
Trade up to the mitochondrial equivalent Prius.
The road to rejuvenation is paved with destruction
For clearing the rubble precedes reconstruction
But remember that life's circular dance
Depends on opposite forces in balance
Excess destruction, too much biogenesis,
Brings heart failure, cancer or neurodegeneries.

Roberta A. Gottlieb

Foreword

It is with great pleasure that I offer a foreword for Volume 4 (Mitophagy), of the Autophagy series edited by M.A. (Eric) Hayat. The series represents a Herculean effort on the part of Professor Hayat. He has recruited an outstanding collection of authors for this volume on mitophagy. Collectively they tell an exciting story of the importance of mitophagy in human pathophysiology.

Early in evolution, eukaryotic cells harnessed mitochondria to capture their efficient energy production from oxidative phosphorylation, but it was equally necessary to establish a mechanism for eliminating them when things went awry. Mitophagy is the elegant pathway for selective autophagic removal of dysfunctional mitochondria, and studies in yeast have and continue to shed light on this complex process. This volume presents the most current understanding of the proteins and pathways involved in mitophagy, including chapters on the selective damage sensors Nix and Bnip3, which respond to mitochondrial reactive oxygen species; PINK1/Parkin, which respond to mitochondrial depolarization; Atg32, which is regulated by phosphorylation; and FUNDC1, which eliminates mitochondria under hypoxic conditions, where they are superfluous and potentially dangerous to the cell. Nine chapters provide an in-depth treatment of the molecular mechanisms involved in mitophagy initiation and execution.

Mitochondrial ATP production is essential to meet the energy requirements of heart and brain. However, the long-lived cells that make up these organs are most vulnerable to the cumulative effects of damaged mitochondria, and as a result rely heavily on mitophagy to maintain optimal organelle function. Ineffective mitophagy manifests in disease affecting these organs before other tissues. Volume 4 includes four chapters on the role of mitophagy in Parkinson disease, cardiac aging, and skeletal muscle atrophy that clearly illustrate the importance of efficient and tightly regulated mitophagy.

Readers will appreciate this comprehensive and up-to-date collection of reviews by many of the scientists who continue to shape the field of mitophagy in human disease. I invite you to delve into this exciting volume, which will doubtless serve as a valuable and contemporary reference.

Roberta A. Gottlieb

Foreword

Intracellular protein turnover was established in the 1940s; before that time, intracellular proteins were considered stable constituents. Christian De Duve discovered lysosomes in the 1950s, and the first electron microscopic images of mitochondria inside lysosomes were published in the late 1950s. The importance of this finding was not fully understood at that time, but now we know that these early micrographs illustrated autophagosomes containing mitochondria. The crucial contribution of lysosomes to the intracellular turnover was finally recognized in the 1970s. Finally, the role of autophagy in the constant recycling of intracellular constituents and organelles was demonstrated in the 1990s, after the discovery of the genes and proteins that regulate autophagy, which has made it possible to monitor and manipulate the autophagic process and to generate knockout and transgenic animal models. This progress is well demonstrated by the fact that in one of the seminal books on intracellular protein degradation, *Lysosomes: Their Role in Protein Degradation* edited by Hans Glaumann and F. John Ballard and published by Academic Press in 1987, the word "autophagy" is mentioned in the title of only two of the twenty chapters. The first book was published in 2003 by Landes Bioscience/Eurekah.com. The first journal devoted to autophagy, also called *Autophagy*, was established in 2005. Since that time, the number of scientific papers and books on autophagy has grown exponentially; also the present book series contributes to the exponential growth. Since the slow start after the discovery of the first autophagosomes by electron microscopy in the 1950s, autophagy finally receives the attention it deserves.

For a long time, autophagy was considered to be nonselective and cytoplasmic constituents and organelles were thought to become randomly sequestered into autophagosomes for the delivery to lysosomes for degradation. Selective autophagy was first discovered in yeast cells, which have several well-known routes for the selective autophagy of different organelles and proteins. The existence of the first molecular mechanisms and the crucial roles of selective autophagy in mammalian cells were in fact an indication of selective removal of aggregate-prone proteins and damaged organelles, including mitochondria, especially in postmitotic cells such as neurons and muscle cells. This volume concentrates on mitophagy, the selective autophagy of mitochondria. Both molecular mechanisms and roles in diseases are addressed by experts in the field.

The field of autophagy still has many unanswered questions to address, and the topic is attracting an increasing number of scientists from different disciplines. This book will be welcomed by the newcomers as a concise overview of the current knowledge on mitophagy. In addition, this volume will also offer the more experienced scientists working on other aspects of autophagy an excellent way to update their knowledge on mitophagy.

Eeva-Liisa Eskelinen

Preface

This is the fourth volume of the series discussing almost all aspects of the autophagy machinery. This volume presents detailed information on the role of mitophagy in health and disease. The most important function of mitochondria is to supply a large amount of energy required for normal cellular activities. This organelle is also involved in a large number of other essential cellular functions, including thermogenesis, iron-sulfur cluster biogenesis, biosynthesis of heme and certain lipids and amino acids, autophagy, apoptosis, immune response, cell death, cellular homeostasis and metabolism, differentiation, aging, and the production of reactive oxygen species (ROS). Therefore, the maintenance of a healthy pool of mitochondria is vital for normal cellular physiology and survival. On the other hand, mitochondrial dysfunction can have severe consequences including aging and pathogenesis of neurodegenerative diseases. In this respect, Parkinson's disease, skeletal muscle atrophy, and cardiovascular disease are discussed here. Various steps involved in mitophagy are detailed, and molecular mechanisms underlying this autophagic machinery are reviewed both in yeast and metazoa. Inclusion of information on autophagy including mitophagy in yeast in this volume is relevant and important because studies of yeast have clarified the fundamental principles of autophagy, which serve as a guide for studies of autophagy in metazoans. Almost all aspects of yeast mitophagy, including proteins involved, generation of reactive oxygen species (ROS), and various mechanisms of mitochondrial quality control, are discussed in detail.

As mentioned above, maintaining a healthy and functional population of mitochondria is critically important for all eukaryotic cells. Several quality control systems exist within mitochondria, and an important link between mitochondria maintenance and macroautophagy (mitophagy) has been established. Mitophagy is one of the primary mechanisms for mitochondrial quality control and serves to selectively eliminate dysfunctional or excess mitochondria via an autophagic process that is tightly regulated. The failure to maintain adequate mitophagy leads to accumulation of dysfunctional mitochondria within cells, resulting in cellular dysfunction. Diseases associated with impaired mitophagy include neurodegenerative diseases, myopathies, obesity, and diabetes, most of which are discussed in this volume. The recent advances in our understanding of mitophagy will provide essential insights into the pathogenesis of a variety of mitochondria dysfunction-related diseases.

Several reviews presenting the current understanding of the molecular mechanisms of autophagy involved in cancer, neurodegeneration, aging, infection, and inflammation are included in this volume. At the molecular level, a large group of proteins has been identified in various model organisms which mediate the association of damaged or dysfunctional mitochondria with the autophagic machinery. Four mammalian mitochondrial proteins (tags) (Nix, PINK1, Bnip3, and FUNDC1) are discussed; also the role of Atg32 protein in yeast is explained. PINK1 (encoded by the *PARK6* gene) and Parkin (encoded by the *PARK2* gene) proteins have provided the

most important insight into the mechanism of autophagy in mammalian cells.

PINK1/Parkin mutants (*Drosophila*) show severe developmental abnormalities associated with mitochondrial dysfunction. In humans, mutations of PINK1 or Parkin are responsible for most cases of early-onset Parkinson's disease. In healthy mitochondria, PINK1 is imported into mitochondrial inner membrane where it is subsequently degraded by PARL, but in mitochondria with disrupted membrane potential, it is retained on the mitochondrial outer membrane where it recruits Parkin from the cytosol. Once recruited, Parkin initiates mitophagy to eliminate dysfunctional mitochondria. The molecular events involved in PINK1/Parkin promotion of mitophagy are detailed in two chapters.

The role of transmembrane protein Atg32 in autophagy is explained in this volume. Phosphorylated Atg32 is an important mitochondrial tag located in the mitochondrial outer membrane. Phosphorylation of Atg32 is required for recruiting the scaffold protein Atg11, resulting in targeting mitochondria for degradation. Independent of Atg11 binding, Atg8 is recruited to Atg32. Atg8 is essential for autophagosome assembly. Atg11 is also required for other types of autophagies. In fact, the formation of a tripartite (Atg32/Atg11/Atg8) initiator complex is common. Casein kinase 2 is essential for the activation of Atg32.

Another example discussed in this volume is the critical role of Nix and related Bnip3 in mitochondrial autophagy. Nix is located in the mitochondrial outer membrane. The transmembrane domain, but not the BH3 domain of Nix, is essential for its activity. Nix is not required for autophagosome formation, but is essential for sequestration of mitochondria into autophagosomes. Nix plays a vital role in the maturation of the reticulocyte to erythrocyte, during which mitochondria are eliminated by mitophagy.

FUNDC1 is a less known protein located in the mitochondrial outer membrane, with structural similarity to Atg32. Hypoxia induces FUNDC1-dependent mitophagy. Mitochondrial fragmentation accompanies FUNDC1-dependent mitophagy. The role of FUNDC1-dependent mitophagy in hypoxic cancer cells is discussed here.

An interesting example of the role of mitophagy is in mammalian reproduction. Mitophagy occurs physiologically during the removal of sperm mitochondria from egg cells upon fertilization; this process is called allophagy. One possible explanation for such selective mitophagy is that paternal mitochondria are heavily damaged by ROS prior to fertilization, and need to be removed to prevent potentially deleterious effects in the next generation.

It is known that the relentless loss of dopaminergic neurons in the midbrain causes Parkinson's disease. Mitochondrial and lysosomal functions decrease with age and, therefore, both are implicated in aging and age-related disorders such as Parkinson's disease. That impaired mitochondrial function is a predominant feature of this disease is explained in this volume. Two specific processes, mitochondrial fission and mitophagy, involved in this disease are described; the former occurs as an early step during neurodegeneration.

As indicated previously, two Parkinson's disease-associated genes, *PINK1* and *Parkin*, are involved in the maintenance of healthy mitochondria. The pivotal role played by Parkin in maintaining dopaminergic neuronal survival is underscored here, and its dysfunction represents a cause of Parkinson's disease. Parkin in cooperation with PINK1 specifically recognizes damaged mitochondria, isolates them from the mitochondrial network, and eliminates them through

the ubiquitin-proteasome and mitophagy pathways. It is emphasized that PINK1 and Parkin protein identify and segregate damaged mitochondria for degradation by mitophagy via ubiquitination of several mitochondrial proteins including mitofusins. Mutations of *PARK2* gene (encoding the ubiquitin ligase Parkin) cause not only familial parkinsonism but also a sporadic form of this disease. As stated before, Parkin is a key regulator of mitochondrial quality control. However, presently the model of Parkin-mediated mitophagy is being debated, which is updated in this volume. The understanding of the molecular mechanisms of PINK1 and Parkin-mediated mitochondrial regulation is also reviewed here.

Intrinsic aging of the cardiovascular system, in addition to chronic exposure to cardiovascular risk factors, is inevitable. This results in the development of cardiovascular disease later in life. It is pointed out that the impairment in mitochondrial function arising from failure of mitochondrial quality control is a major contributing factor to heart senescence. It is also pointed out that damaged mitochondria produce increased amounts of ROS, resulting in oxidative damage to cardiomyocyte components.

Loss of muscle mass and function results mostly from accelerated protein degradation by the ubiquitin-proteasome system and autophagy-lysosome systems. The signaling mechanism underlying the increased protein degradation during muscle atrophy from a genetic perspective is explained here. The importance of mitophagy during skeletal muscle atrophy is pointed out.

The text is divided into three subheadings (General Applications, Molecular Mechanisms, and Role in Disease) for the convenience of the readers.

By bringing together a large number of experts (oncologists, physicians, medical research scientists, and pathologists) in the field of mitophagy, it is my hope that substantial progress will be made against terrible diseases afflicting humans. It is difficult for a single author to discuss effectively and comprehensively various aspects of an exceedingly complex process such as mitophagy. Another advantage of involving more than one author is to present different points of view on various controversial aspects of the role of mitophagy in health and disease. I hope these goals will be fulfilled in this and future volumes of this series.

This volume was written by 39 contributors representing 9 countries. I am grateful to them for their promptness in accepting my suggestions. Their practical experience highlights the very high quality of their writings, which should build and further the endeavors of the readers in this important medical field. I respect and appreciate the hard work and exceptional insight into the mitophagy machinery provided by these contributors.

It is my hope that subsequent volumes of this series will join this volume in assisting in the more complete understanding of the complex process of autophagy, and eventually in the development of therapeutic applications. There exists a tremendous, urgent demand by the public and the scientific community to develop better treatments for major diseases. In the light of the human impact of these untreated diseases, government funding must give priority to researching cures over global military superiority.

I am grateful to Dr. Dawood Farahi, Phillip Connelly, and Dr. Veysel Yucetepe for recognizing the importance of medical research and publishing through an institution of higher education. I am thankful to my students for their contributions to the final preparation of this volume.

M. A. Hayat
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Abbreviations and Glossary

1AP	inhibitor of apoptosis protein
3-MA	3-methyladenine, an autophagy inhibitor
3-methyladenine	an autophagic inhibitor
5-Fu	5 fluorouracil
AAP	protein that mediates selective autophagy
ACF	aberrant crypt foci
aggrephagy	degradation of ubiquitinated protein aggregates
aggresome	inclusion body where misfolded proteins are confined and degraded by autophagy
AIF	apoptosis-inducing factor
AIM	Atg8-family interacting motif
Akt	a.k.a. protein kinase B; regulates autophagy
Alfy	autophagy-linked FYVE protein
ALIS	aggresome-like induced structures
ALR	autophagic lysosome reformation
AMBRA-1	activating molecule in Beclin 1-regulated autophagy
AMP	adenosine monophosphate
amphisome	intermediate compartment formed by fusing an autophagosome with an endosome
AMPK	adenosine monophosphate-activated protein kinase
APC	antigen-presenting cells
APG	autophagy
aPKC	atypical protein kinase C
APMA	autophagic macrophage activation
apoptosis	programmed cell death type 1
ARD1	arrest-defective protein 1
ASK	apoptosis signal regulating kinase
AT1	Atg8-interacting protein
ATF5	activating transcription factor 5
ATF6	activating transcription factor 6
Atg	autophagy-related gene or protein
Atg1	serine/threonine protein 1 kinase
Atg2	protein that functions along with Atg18
Atg3	ubiqitin conjugating enzyme analogue
Atg4	cysteine protease
Atg5	protein containing ubiquitin folds

Atg6	component of the class III PtdIns 3-kinase complex
Atg7	ubiquitin activating enzyme homologue
Atg8	ubiquitin-like protein
Atg9	transmembrane protein
Atg10	ubiquitin conjugating enzyme analogue
Atg11	fungal scaffold protein
Atg12	ubiquitin-like protein
Atg13	component of the Atg1 complex
Atg14	component of the class III PtdIns 3-kinase complex
Atg15	vacuolar protein
Atg16	component of the Atg12-Atg5-Atg16 complex
Atg17	yeast protein
Atg18	protein that binds to PtdIns
Atg19	receptor for the Cvt pathway
Atg20	PtdIns P binding protein
Atg21	PtdIns P binding protein
Atg22	vacuolar amino acid permease
Atg23	yeast protein
Atg24	PtdIns binding protein
Atg25	coiled-coil protein
Atg26	sterol glucosyltransferase
Atg27	integral membrane protein
Atg28	coiled-coil protein
Atg29	protein in fungi
Atg30	protein required for recognizing peroxisomes
Atg31	protein in fungi
Atg32	mitochondrial outer membrane protein
Atg33	mitochondrial outer membrane protein
Atg101	Atg13 binding protein
ATM	ataxia-telangiectasia mutated protein
autolysosome protein	lysosomal associated membrane protein 2
autolysosome	formed by fusion of the autophagosome and lysosome, degrading the engulfed cell components
autophagic body	the inner membrane-bound structure of the autophogosome
autophagic flux	the rate of cargo delivery to lysosomes through autophagy
autophagosome	double-membrane vesicle that engulfs cytoplasmic contents for delivery to the lysosome
autophagosome maturations	events occurring post-autophagosome closure followed by delivery of the cargo to lysosomes
autophagy	programmed cell death type 2
AV	autophagic vacuole
axonopathy	degradation of axons in neurodegeneration
BAD	Bcl-2 associated death promoter protein
Bafilomycin	inhibitor of the vacular-type ATPase
Bafilomycin A1(Baf-A1)	an autophagy inhibitor
BAG	Bcl-2-associated athanogene

BAG3	Bcl-2-associated athanogene 3
BAK	Bcl-2 antagonist/killer
Barkor	Beclin 1-associated autophagy-related key regulator
BATS	Barkor/Atg14(L) autophagosome targeting sequence
BAX	Bcl-2-associated X protein
Bcl-2	B cell lymphoma-2
Beclin 1	mammalian homologue of yeast Atg6, activating macroautophagy
Beclin 1	Bcl-2-interacting protein 1
BH3	Bcl-2 homology domain-3
BH3-only proteins	induce macroautopagy
BHMT	betaine homocysteine methyltransferase protein found in the mammalian autophagosome (metabolic enzyme)
BID	BH3-interacting domain death agonist
Bif-1 protein	interacts with Beclin 1, required for macroautophagy
Bim	Bcl-2 interacting mediator
BNIP	pro-apoptotic protein
BNIP3 protein	required for the HIF-1-dependent induction of macroautophagy
bortezomib	selective proteasome inhibitor
CaMKKβ protein	activates AMPK at increased cytosolic calcium concentration
CaMK	calcium/calmodulin-dependent protein kinase
CASA	chaperone-assisted selective autophagy
caspase	cysteine aspartic acid specific protease
CCI-779	rapamycin ester that induces macroautophagy
CD46 glycoprotein	mediates an immune response to invasive pathogens
chloroquine	an autophagy inhibitor which inhibits fusion between autophagosomes and lysosomes
c-Jun	mammalian transcription factor that inhibits starvation-induced macroautophagy
Clg 1	a yeast cyclin-like protein that induces macroautophagy
CMA	chaperone-mediated autophagy
COG	functions in the fusion of vesicles within the Golgi complex
COP1	coat protein complex1
CP	20S core particle
CRD	cysteine-rich domain
CSC	cancer stem cell
CTGF	connective tissue growth factor
Cvt	cytoplasm-to-vacuole targeting
DAMP	damage-associated molecular pattern molecule/danger-associated molecular pattern molecule
DAP1	death-associated protein 1
DAPK	death-associated protein kinase
DAPK1	death-associated protein kinase 1
DDR	DNA damage response
DEPTOR	DEP domain containing mTOR-interacting protein
DFCP1	a PtdIns (3) P-binding protein

DISC death-inducing signaling complex
DMV double-membrane vesicle
DOR diabetes-and obesity-regulated gene
DRAM damage-regulated autophagy modulator
DRAM-1 damage-regulated autophagy modulator 1 induces autophagy in a p53-dependent manner.
DRC desmin-related cardiomyopathy
DRiP defective ribosomal protein
DRP1 dynamin related protein 1
DUB deubiquitinases that accumulate proteins into aggresomes
E2F1 a mammalian transcription factor
efferocytosis phagocytosis of apoptotic cells
EGFR epidermal growth factor receptor
EIF2α eukaryotic initiation factor 2 alpha kinase
endosomes early compartments fuse with autophagosomes to generate amphisomes
ERAA endoplasmic reticulum-activated autophagy
ERAD endoplasmic reticulum-associated degradation pathway
ERK extracellular signal regulated kinase
ERK1/2 extracellular signal regulated kinase 1/2
ERT enzyme replacement therapy
ESCRT endosomal sorting complex required for transport
everolimus mTOR inhibitor
FADD Fas-associated death domain
FKBP12 FK506-binding protein 12
FoxO3 Forkhead box O transcription factor 3
FYCO1 FYVE and coiled domain containing 1
GAA acid α-glucosidase
GABARAP gamma-aminobutyric acid receptor-associated protein
GAS group A streptococcus
GATE-16 Golgi-associated ATPase enhancer of 16 kDa
GFP green fluorescent protein
glycophagy degradation of glycogen particles
GPCR G protein-coupled receptor
GSK-3β glycogen synthase kinase 3 beta; regulates macroautophagy
GST-BHMT BHMT fusion protein used to assay macroautophagy in mammalian cells
HAV heavy autophagic vacuole
HCQ hydroxychloroquine
HCV hepatitis C virus
HDAC histone deacetylase
HDAC6 histone deacetylase 6
HIF hypoxia-inducible factor
HIF1 hypoxia-inducible factor 1
HMGB1 high mobility group box 1

HR-PCD	hypersensitive response programmed cell death
Hsc70	heat shock cognate protein
HSP	heat shock protein
Hsp90	heat shock protein 90
HspB8	heat shock cognate protein beta-8
Htraz	high temperature requirement factor Az; a pro-apoptotic protein
I13P	phosphatidylinositol
IAP	inhibitor of apoptosis protein
IKK	inhibitor of nuclear factor κB
IL3	interleukin-3
IM	isolation membrane
inflammasome	an intracellular protein complex that activates caspase-1
IRF	interferon regulatory factor
IRGM	immunity-associated GTPase family M
IRS	insulin receptor substrate
JNK/SAPK	c-Jun N-terminal kinase/stress-activated protein kinase
KRAS	an oncogene that induces autophagy in cancer cells
LAMP	lysosome-associated membrane protein
LAMP1	lysosome marker, lysosome-associated membrane protein 1
LAMP2	lysosomal-associated membrane protein 2
LAMP-2A	lysosomal-associated membrane protein 2A
LAP	LC3-associated phagocytosis
LAV	light autophagic vacole
LC3 (MAP1LC3B)	autophagosome marker microtubule-associated protein 1 light chain 3B
LC3	microtubule-associated protein light chain 3
LET	linear energy transfer
lipophagy	selective delivery of lipid droplets for lysosomal degradation
LIR	LC3 interacting region
LKB	liver kinase B
LSD	lysosomal storage disorder
lysosomotropic agent	compound that accumulates preferentially in lysosomes
macroautophagy	autophagy
macrolipophagy	regulation of lipid metabolism by autophagy
MALS	macroautophagy–lysosome system
MAPK	mitogen-activated protein kinase
MARF	mitofusion mitochondrial assembly regulatory factor
MCU	mitochondrial calcium uptake uniporter pore
MDC	monodansylcadaverine to measure autophagic flux *in vivo*
MEF	mouse embryonic fibroblast
MFN2	mitofusin 2, a mitochondrial outer membrane protein involved in fusion/fission to promote mitochondrial segregation and elimination
MHC	major histocompatibility complex

MHC-II	major histocompatibility complex class II
MiCa	mitochondrial inner membrane calcium channel
micropexophagy or macropexophagy	peroxisome degradation by autophagic machinery
MIPA	micropexophagy-specific membrane apparatus
mitofusion	mitochondrial fusion-promoting factor
mitophagy	degradation of dysfunctional mitochondria
MOM	mitochondrial outer membrane
MPS	mucopolysaccharide
MPT	mitochondrial permeability transition
mPTP	mitochondrial permeability transition pore
MSD	multiple sulfatase deficiency
MTCO2	mitochondrial marker
MTOC	microtubule organizing center
mTOR	mammalian target of rapamycin, which inhibits autophagy and functions as a sensor for cellular energy and amino acid levels
mTORc1	mammalian target of rapamycin complex 1
MTP	mitochondrial transmembrane potential
MTS	mitochondrial targeting sequence
MVB	multivesicular body
NBR1	neighbor of BRCA1 gene 1
NDP52	nuclear dot protein 52 kDa
NEC-1	necrostatin-1
necroptosis	a form of programmed cell death by activating autophagy-dependent necrosis
Nix	a member of the Bcl-2 family required for mitophagy
NLR	NOD-like receptor
NOD	nucleotide-binding oligomerization domain
NOS	nitric oxide synthase
NOX	NADPH oxidase
Nrf2	nuclear factor 2
OCR	oxygen consumption rate
Omegasome	PI(3)P-enriched subdomain of the ER involved in autophagosome formation
OMM	outer mitochondrial membrane
OPA1	dynamin-related protein; requires mitafusin 1 to promote mitochondrial fusion
Ox-LDL	oxidized low density lipoprotein is a major inducer of ROS, inflammation, and injury to endothelial cells
p62	an autophagy substrate
p62/SQSTM1	sequestosome 1
PAMP	pathogen-associated molecular pattern molecule
PAS	pre-autophagosomal structure
PB1 domain	Phox and Bem1 domain
PCD	programmed cell death

PDI	protein disulfide isomerase
PE	phosphatidylethanolamine
PERK	protein kinase-like endoplasmic reticulum kinase
PFI	proteasome functional insufficiency
Phagophore	a cup-shaped, double membraned autophagic precursor structure
PI(3)K-PKB-FOXO	a growth factor that inhibits autophagy and increases apoptosis by regulating glutamine metabolism
PI3K	phosphatidylinositol 3-kinase
PI3KC3	phosphatidylinositol-3-kinase class III
PINK1	PTEN (phosphatase and tensin homologue deleted on chromosome 10)-induced putative kinase 1
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
polyQ	polyglutamine
PQC	protein quality control
prion disease	transmissible spongiform encephalopathy
PRR	pathogen recognition receptor
PS	phosphatidyl serine
PSMB5	proteasome subunit beta type-5
PtdIns	phosphatidylinositol
PTGS	post-transcriptional gene silencing
PUMA	p53 upregulated modulator of apoptosis
R1G	retrograde signaling pathway
Rag	GTPase that activates TORC1 in response to amino acids
RAGE	receptor for advanced glycation end product
rapamycin	a well-known autophagy inducer by suppressing mTOR
RAPTOR	regulatory-associated of mTOR
RE	recycling endosome
residual body	lysosome containing undegraded material
reticulophagy	degradation of endoplasmic reticulum
ribophagy	degradation of ribosomes
RIP	receptor-interacting protein
RISC	RNA-induced silencing complex
RLS	reactive lipid species
RNAi	RNA interference
RNS	reactive nitrogen species
ROS	reactive oxygen species
ROT	rottlerin; used as a protein kinase C-delta inhibitor
RP	19S regulatory particle
Rubicon	RUN domain and cysteine-rich domain-containing Beclin 1-interacting protein
selective autophagy	selective recruitment of substrates for autophagy
sequestosome 1	an autophagy substrate

sequestosome 1 (p62/SQSTM1)	a multifunctional adapter protein implicated in tumorigenesis
sequestosome (SQSTMI)1	p62 protein; a ubiquitin-binding scaffold protein
SESN2	sestrin-2
shRNA	small/short hairpin RNA
siRNA	small interference RNA
sirt 1	sirtuin 1 class III histone deacetylase, prevents Alzheimer's disease
SMIR	small molecule inhibitor of rapamycin
SNARE	soluble N-ethylmaleimide-sensitive factor attachment receptor
SNP	single nucleotide polymorphism
SQSTM1	sequestosome 1
SVR	sustained virological response
Syt1	synaptotagmin1
T1DM	type 1 diabetes mellitus
TAKA	transport of Atg9 after knocking-out Atg1
TASCC	TOR-autophagy spatial coupling compartment
TCN	transe-Golgi network
TCR	T cell receptor
TECPR1	tectonin beta-propeller repeat containing 1
Tensirolimus	mTOR inhibitor
TFEB	transcript factor EB
TGFβ	transforming growth factor β that activates autophagy
TGN	trans-Golgi network
TIGR	TP53 (tumor protein 53)-induced glycolysis and apoptosis regulator
TK	tyrosine kinase
TKI	tyrosine kinase inhibitor
TLR	Toll-like receptor
TMD	transmembrane domain
TMEM166	transmembrane protein 166 that induces autophagy
TNF	tumor-necrosis factor
TNF-α	tumor necrosis factor alpha
Torin1	ATP-competitive mTOR inhibitor
TRAIL	tumor necrosis factor-regulated apoptosis-inducing ligand
TSC	tuberous sclerosis complex
TSC2	tuberous sclerosis complex 2
TSP	thrombospondin
UBA domain	ubiquitin-associated domain
UBAN	ubiquitin-binding domain
ubiquitin	a small protein that functions in intracellular protein breakdown and histone modification
ubiquitination	a well-established signal for inducing autophagy of protein aggregates

Ubl	ubiquitin-like
ULK	Unc-51-like kinase complex
ULK1	putative mammalian homologue of Atg1p
UPR	unfolded protein response
UPS	ubiquitin–proteasome system
UVRAG	UV-irradiation resistance-associated gene
VAchT	vesicular acetylcholine transporter
VAMP	vesicle-associated membrane protein
VCP/p97	valosin-containing protein involved in endosomal trafficking and autophagy
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VMP1	vacuole membrane protein 1; promotes formation of autophagosomes
VPS15	vacuolar protein sorting 15 homologue
VTA	vascular targeting agent
VTC	vacuolar transporter chaperone
wortmannin	an autophagic inhibitor
XBP1	a component of the ER stress response that activates macroautophagy
xenophagy	degradation of invading bacteria, viruses, and parasites
YFP	yellow fluorescent protein
zymophagy	lysosomal degradation of zymogen granules (digestive enzymes)

See also Klionsky, D. J., Codogno, P., Cuervo, A. M. *et al*. (2010). A comprehensive glossary of autophagy-related molecules and processes. *Autophagy* 6, 438–448.

Autophagy: Volume 1 – Contributions

Autophagy: Volume 2 – Contributions

Autophagy: Volume 3 – Contributions

CHAPTER

1

Introduction to Autophagy: Cancer, Other Pathologies, Inflammation, Immunity, Infection, and Aging, Volume 4

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OUTLINE

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Abstract

Autophagy plays a direct or indirect role in health and disease. A simplified definition of autophagy is that it is an exceedingly complex process which degrades modified, superfluous (surplus) or damaged cellular macromolecules and whole organelles using hydrolytic enzymes in the lysosomes. It consists of sequential steps of induction of autophagy, formation of autophagosome precursor, formation of autophagosome, fusion between autophagosome and lysosome, degradation of cargo contents, efflux transportation of degraded products to the cytoplasm, and lysosome reformation.

This chapter discusses specific functions of autophagy, the process of autophagy, major types of autophagy, influences on autophagy, and the role of autophagy in disease, immunity, and defense.

INTRODUCTION

Aging has so permeated our lives that it cannot be stopped, but it can be delayed. Under the circumstances, time is our only friend. Because the aging process is accompanied by disability and disease (for example, Alzheimer's and Parkinson's conditions) and cannot be prevented, it seems that slow aging is the only way to have a healthy longer life. In general, aging can be slowed down by not smoking or chewing tobacco, by preventing or minimizing perpetual stress (anger, competition), by abstinence from alcoholic beverages, by regular exercise, and by having a healthy diet. There is no doubt that regular physical activity is associated with a reduced risk of mortality and contributes to the primary and secondary prevention of many types of diseases. Discipline is required to attain this goal.

Regarding the role of a healthy diet, a caloric restriction induces autophagy that counteracts the development of age-related diseases and aging itself. On the other hand, autophagy

is inhibited by high glucose and insulin-induced P13K signaling via Akt and mTOR. Based on its fundamental roles in these and other disease processes' prevention and therapy, autophagy has emerged as a potential target for disease.

Unfortunately, inevitable death rules our lives, and a group of abnormal cells plays a part in it. Safe disposal of cellular debris is crucial to keep us alive and healthy. Our body uses autophagy and apoptosis as clearing mechanisms to eliminate malfunctioning, aged, damaged, excessive, and/or pathogen-infected cell debris that might otherwise be harmful/autoimmunogenic. However, if such a clearing process becomes uncontrollable, it can instead be deleterious. For example, deficits in protein clearance in the brain cells because of dysfunctional autophagy may lead to dementia. Autophagy can also promote cell death through excessive self-digestion and degradation of essential cellular constituents.

Humans and other mammals with long lifespans unfortunately have to face the problem of the accumulation of somatic mutations over time. Although most of the mutations are benign and only some lead to disease, there are too many of them. Cancer is one of these major diseases, and is caused by a combination of somatic genetic alterations in a single cell, followed by uncontrolled cell growth and proliferation. Even a single germline deletion of or mutation in a tumor suppressor gene (e.g., *p53*) predisposes an individual to cancer. It is apparent that nature tries to ensure the longevity of the individual by providing tumor suppressor genes and other protective mechanisms. Autophagy (*Beclin 1* gene) is one of these mechanisms that plays an important role in influencing the aging process.

Autophagy research is in an explosive phase, driven by a relatively new awareness of the enormously significant role it plays in health and disease, including cancer, other pathologies, inflammation, immunity, infection, and aging. The term autophagy (*auto phagin*, from the Greek meaning self-eating) refers to a phenomenon in which cytoplasmic components are delivered to the lysosomes for bulk or selective degradation under the lysosomes' distinct intracellular and extracellular milieu. This term was first coined by de Duve over 46 years ago (Deter and de Duve, 1967), based on the observed degradation of mitochondria and other intracellular structures within lysosomes of rat liver perfused with the pancreatic hormone, glucagon.

Over the past two decades an astonishing advance has been made in the understanding of the molecular mechanisms involved in the degradation of intracellular proteins in yeast vacuoles and the lysosomal compartment in mammalian cells. Advances in genome-scale approaches and computational tools have presented opportunities to explore the broader context in which autophagy is regulated at the systems level.

A simplified definition of autophagy is that it is an exceedingly complex process which degrades modified, superfluous (surplus), or damaged cellular macromolecules and whole organelles using hydrolytic enzymes in the lysosomes. Autophagy can be defined in more detail as a regulated process of degradation and recycling of cellular constituents participating in organelle turnover, resulting in the bioenergetic management of starvation. This definition, however, still represents only some of the numerous roles played by the autophagic machinery in mammals; most of the autophagic functions are listed later in this chapter.

Autophagy plays a constitutive and basally active role in the quality control of proteins and organelles, and is associated with either cell survival or cell death. Stress-responsive autophagy can enable adaptation and promote cell survival, whereas in certain models, autophagy has also been associated with cell death, representing either a failed attempt at

survival or shown to be a mechanism that supports cell and tissue degradation. Autophagy prevents the accumulation of random molecular damage in long-lived structures, particularly mitochondria, and more generally provides a means to reallocate cellular resources from one biochemical pathway to another. Consequently, it is upregulated in conditions where a cell is responding to stress signals, such as starvation, oxidative stress, and exercise-induced adaptation. The balance between protein and lipid biosynthesis, and their eventual degradation and resynthesis, is one critical component of cellular health.

Degradation and recycling of macromolecules via autophagy provides a source of building blocks (amino acids, fatty acids, sugars) that allow temporal adaptation of cells to adverse conditions. In addition to recycling, autophagy is required for the degradation of damaged or toxic material that can be generated as a result of ROS accumulation during oxidative stress. The mitochondrial electron transport chain and the peroxisomes are primary sources of ROS production in most eukaryotes.

SPECIFIC FUNCTIONS OF AUTOPHAGY (A SUMMARY)

Autophagy plays a direct or indirect role in health and disease, including, among others, control of embryonic and early postnatal development; tissue homeostasis (protein and cell organelle turnover); mitochondrial quality control; protection of cells from stresses; survival response to nutrient deprivation; cellular survival or physiological cell death during development; involvement in cell death upon treatment with chemotherapy and radiotherapy; tissue remodeling during differentiation and development, including regulation of number of cells and cell size, endocytosed gap junctions, villous trophoblasts, cellular house-cleaning, protein, glucose, and lipid metabolism; supply of energy; anti-aging; human malignancy, tumorigenesis, tumor maintenance, inflammation, cancer (pro and anti), ovarian cancer, nasopharyngeal carcinoma, melanoma, colon cancer, and neutrophil differentiation of acute promyelocytic leukemia; lysosomal storage diseases; metabolic disorders; osteoarthritis; cardiovascular diseases; alcoholic cardiomyopathy, and steatosis in alcoholics (fatty degeneration of the heart); neurodegenerative diseases (Alzheimer's, Parkinson's, Huntington's, amyotrophic lateral sclerosis, and prion disease); muscular dystrophy; skeletal myopathy; atherosclerosis; diabetes; obesity; lipid degradation in the liver; alcoholic liver disease; pancreatitis; cellular quality control; protection of the genome; innate and adoptive immune responses to infection by microbial pathogens; defense against intracellular bacterial, parasitic, and viral infections; protection of intracellular pathogens; epileptogenesis; Pompe disease; nephropathy; reduction of liver damage during ischemia–reperfusion; regression of the corpus luteum; protection of stem cells from apoptosis during stress; and cross-talk with apoptosis, among other functions. Neonates also adapt to transitive starvation by inducing autophagy.

AUTOPHAGY IN NORMAL MAMMALIAN CELLS

Although autophagy mediates cell adaptation to a range of stress conditions, including starvation, this stress is not a problem that a normal cell of a multicellular organism

would face on a regular basis. The basal level of autophagy (the so-called basal or quality control autophagy) is found in most cells, and is required for the normal clearance of potentially deleterious protein aggregates that can cause cellular dysfunction. Thus, mammalian autophagy is primarily required for intracellular cleaning of misfolded proteins and damaged/old organelles. In the absence of such cleaning, neoplastic transformation is likely.

As alluded to above, starvation is uncommon in mammalian cells under normal nutritional conditions. Therefore, it is important to know the mechanism responsible for regulating autophagy under normal nutritional conditions. In mammalian cells, mTOR kinase, the target of rapamycin, mediates a major inhibitory signal that represses autophagy under nutrient-rich conditions. Calpain 1 keeps autophagy under tight control by downregulating the levels of Atg12–Atg5 conjugate. Atg5 and Atg12–Atg5 conjugate are key signaling molecules for increasing the levels of autophagy (Xia *et al*., 2010). It is also known that intracellular Ca^{2+} regulates autophagy. Inhibition of Ca^{2+} influx results in the induction of autophagy. Reduction in intracellular Ca^{2+} prevents the cleavage of Atg5, which in turn increases the levels of full-length Atg5 and Atg12–Atg5 conjugate. The Atg12–Atg5 signaling molecule is regulated by calpain 1 in controlling the levels of autophagy in mammalian cells under nutrient-rich conditions. It is known that inhibition of calpains induces autophagy, and reduces the accumulation of misfolded proteins. It is further known that increased levels of LC3-II in fluspirilene-treated cells promote autophagy by increasing the levels of Atg5 and Atg12–Atg5 conjugate; fluspirilene is one of the autophagy inducers. Although autophagy is maintained at very low levels in normal mammalian cells, it can be rapidly induced within minutes upon starvation, or invasion by intracellular pathogens.

MAJOR TYPES OF AUTOPHAGIES

Based on the type of cargo delivery, there are three types of autophagy systems in mammals – macroautophagy (autophagy), microautophagy, and chaperone-mediated autophagy – each of which is discussed below. Although significant advances (some of which are included here) have been made in our understanding of different types of autophagies, many unanswered questions remain. A further understanding of the exact functions of the three types of autophagy is necessary before we can manipulate these pathways to treat human diseases.

Macroautophagy (Autophagy)

Whole regions of the cytosol are sequestered and delivered to lysosomes for degradation. Cargo sequestration occurs in the autophagosome, a double-membrane vesicle that forms through the elongation and sealing of a *de novo* generated membrane (Ohsumi and Mizushima, 2004). This limiting membrane originates from a tightly controlled series of interactions between more than 10 different proteins which resemble the conjugation steps that mediate protein ubiquitinization (Cuervo, 2009). Formation of the limiting membrane also requires the interaction between a protein and a specific lipid molecule, regulated by conjugating enzymes.

Microautophagy

Microautophagy is the direct uptake of soluble or particulate cellular constituents into lysosomes. It translocates cytoplasmic substances into the lysosomes for degradation via direct invagination, protrusion, or septation of the lysosomal limiting membrane. In other words, microautophagy involves direct invagination and fusion of the vacuolar/lysosomal membrane under nutrient limitation. The limiting/sequestering membrane is the lysosomal membrane, which invaginates to form tubules that pinch off into the lysosomal lumen.

Microautophagy of soluble components, as in macroautophagy (autophagy), is induced by nitrogen starvation and rapamycin. Microautophagy is controlled by the TOR and EGO signaling complexes, resulting in direct uptake and degradation of the vacuolar boundary membrane (Uttenweiler *et al*., 2007). Hence, this process could compensate for the enormous influx of membrane caused by autophagy.

It seems that microautophagy is required for the maintenance of organelle size and membrane composition rather than for cell survival under nutrient restriction. Uttenweiler *et al*. (2007) have identified the vacuolar transporter chaperone, VTC complex, required for microautophagy. This complex is present on the endoplasmic reticulum and vacuoles, and at the cell periphery. Deletion of the VTC complex blocks microautophagic uptake into vacuoles.

Chaperone-Mediated Autophagy

Chaperone-mediated autophagy (CMA) is a generalized form of autophagy present in almost all cell and tissue types. It has been characterized in higher eukaryotes but not in yeast. Because of the particular characteristics of this type of delivery, explained below, only soluble proteins, but not whole organelles, can be degraded through CMA (Cuervo, 2009). CMA is dependent on the constitutively expressed heat shock cognate 70 (Hsc70), shares 80% homology with the heat shock protein 70 (Hsp70), and identifies peptide sequences of cytoplasmic substrates; thus, it is more selective than autophagy in its degradation (Hoffman *et al*., 2012). CMA serves to balance dysregulated energy, and is maximally activated by nutrient/metabolic and oxidative/nitrostative stresses. Cross-talk between CMA and autophagy is likely. CMA differs from the other two types of autophagies with respect to the mechanism for cargo selection and delivery to the lysosomal lumen for degradation. In other words, CMA is involved in the delivery of cargo, which does not require the formation of intermediate vesicles, membrane fusion, or membrane deformity of any type. Instead, the substrates are translocated from the cytosol directly into the lysosomal lumen across the membrane in a process mediated by a translocation protein complex that requires the substrate unfolding.

A chaperone protein binds first to its cytosolic target substrate, followed by a receptor on the lysosomal membrane at the site of protein unfolding. This protein is subsequently translocated into the lysosome for its degradation. In this system the substrate proteins are selectively targeted one-by-one to the lysosomes, and are then translocated across the lysosomal membrane. This selectivity and direct lysosomal translocation have thus become trademarks of CMA.

All the CMA substrate proteins are soluble cystolic proteins. An essential requirement for a protein to become a CMA substrate is the presence of a pentapeptide motif, biochemically

related to KFERQ in its amino acid sequence (Dice, 1990). The motif present in ~30% of the proteins in the cytosol, is recognized by a cytosolic chaperone, the heat shock cognate protein of 73 kDa (cyt-Hsc70). The interaction with chaperone, modulated by the Hsc70 co-chaperones, targets the substrate to the lysosomal membrane, where it interacts with the lysosomal membrane protein (LAMP) type 2a (Cuervo and Dice, 1996). During CMS, proteins are directly imported into lysosomes via the LAMP-2a transporter assisted by the cytosolic and lysosomal HSC70 chaperone that recognizes the KFERG-like motif. Substrates of CMA carry signal peptides for sorting into lysosomes, similar to other protein-transport mechanisms across membranes. Substrates are required to be unfolded before translocation into the lysosomal lumen. Several cytosolic chaperones associated with the lysosomal membrane have been proposed, which assist in the unfolding (Aggarraberes and Dice, 2001). Translocation of the substrate requires the presence of a variant of Hsc70, lys-Hsc70, in the lysosomal lumen. This is followed by the rapid proteolysis of the substrate by residual lysosomal proteases (half-life of 5–10 minutes in the lysosomal lumen).

AUTOPHAGOSOME FORMATION

Autophagy is a highly complex process consisting of sequential steps of induction of autophagy, formation of autophagosome precursor, formation of autophagosome, fusion between autophagosome and lysosome, degradation of cargo contents, efflux transportation of degraded products to the cytoplasm, and lysosome reformation.

In mammalian cells autophagosome formation begins with a nucleation step, where isolation membranes of varied origins form phagophores which then expand and fuse to form a completed double-membrane vesicle called an autophagosome (Luo and Rubinsztein, 2010). Autophagosomes are formed at random sites in the cytoplasm. They move along microtubules in a dynein-dependent fashion towards the microtubule-organizing center, where they encounter lysosomes. After fusion with lysosomes the cargo is degraded with hydrolases, followed by the reformation of lysosomes primarily by the Golgi complex.

The isolation membranes may be generated from multiple sources that include endoplasmic reticulum (ER), Golgi complex, outer mitochondrial membrane, and plasma membrane; however, the ER source is more feasible because it, along with its ribosomes, is involved in protein synthesis. The presence of many Atg proteins near the ER also suggests that ER plays an important role as a membrane source for autophagosome formation. The formation of isolation membrane is initiated by class III phosphatidylinositol 3-kinase (PI3KC)/Beclin 1-containing complexes. Elongation of the isolation membrane involves two ubiquitin-like conjugation systems. In one of them, Atg12 associates with Atg5 to form Atg12–Atg5–Atg16L1 molecular complexes that bind the outer membrane of the isolation membrane. In the second, lipidated microtubule-associated light chain 3 (LC3) is conjugated to phosphatidylethanolamine to generate a lipidated LC3-II form, which is integrated in both the outer and inner membranes of the autophagosome (Fujita *et al.*, 2008). Recently, it was reported that human Atg2 homologues Atg2A and AtgB are also essential for autophagosome formation, presumably at a late stage (Velikkakath *et al.*, 2012).

Autophagosome membrane formation requires autophagy-related proteins (Atgs) along with the insertion of lipidated microtubule-associated light chain 3 (LC3) or gamma-aminobutyric

acid A receptor-associated protein (GABARAP) subfamily members. Various components in the autophagosomal compartment can be recognized by the presence of specific autophagy molecules. Atg16L1 and Atg5 are mainly present in the phagophore, while LC3 labels isolation membranes, matured autophagosomes, and autolysosomes (Gao *et al.*, 2010). This evidence suggests that different Atg molecules participate in autophagosome biogenesis at various stages. Autophagosome substrate selectivity can be conferred by interactions between LC3 and specific cargo receptors, including sequestosome-1 (SQSTM1 #p62) and a neighbor of BRCA1 (NBR1). During this process of autophagy, both lipidated LC3 (LC3-II) and the cargo receptors are degraded (Hocking *et al.*, 2012).

In yeast, the Atg5–Atg12/Atg16 complex is essential for autophagosome formation (Romanov *et al.*, 2012). This complex directly binds membranes. Membrane binding is mediated by Atg5, inhibited by Atg12, and activated by Atg16. All components of this complex are required for efficient promotion of Atg8 conjugation to phosphatidylethanolamine. However, this complex is able to tether (fasten) membranes independently of Atg8.

AUTOPHAGIC LYSOSOME REFORMATION

Following degradation of engulfed substrates with lysosomal hydrolytic enzymes and release of the resulting molecules (amino acids, fatty acids, monosaccharides, nucleotides), autophagic lysosome reformation (ALR) occurs. Although a great deal is known regarding the molecular mechanisms involved in the formation of autophagosomes and autolysosomes, the available information on post-degradation events, including ALR, is inadequate. The importance of such information becomes apparent considering that autophagosomes can fuse with multiple lysosomes. Thus, post-degradation of substrates might result in the depletion of free lysosomes within a cell unless free lysosomes are rapidly reformed. A cellular mechanism is required for maintaining lysosome homeostasis during and after autophagy.

Some information is available at the molecular level regarding the process of ALR. The ALR process can be divided into six steps (Chen and Yu, 2012): phospholipid conversion, cargo sorting, autophagosomal membrane budding, tubule extension, budding and fusion of vesicles, and protolysosome maturation. Initially, LAMP1-positive tubular structures extend from the autolysosomes; these appear empty, without detectable luminal contents from the autolysosomes. Lysosomal membrane proteins (LAMP1, LAMP2) only are located on these tubules; autophagosomal membrane proteins (LC3) are absent.

The role of mTOR is also relevant in the ALR. It has been found that the starvation-induced autophagy process is transient. During starvation, intracellular mTOR is inhibited before autophagy can occur, but it is reactivated after prolonged starvation, and the timing of this reactivation is correlated with the initiation of ALR and termination of autophagy (Chen and Yu, 2012). Thus, mTOR reactivation is required for ALR. ALR is blocked when mTOR is inhibited, and mTOR reactivation is linked to lysosomal degradation.

The lysosomal efflux transporter spinster is also required to trigger ALR (Rong *et al.*, 2011); these transporters are lysosomal membrane proteins that export lysosomal degradation products. Sugar transporter activity of spinster is essential for ALR. Inhibition of spinster results in the accumulation of a large amount of undigested cytosol in enlarged

autolysosomes, seen in the transmission electron microscope, as a result of over-acidification of autolysosomes (Rong *et al*., 2011).

Clathrin is also essential for ALR. It is known that clathrin proteins play an important role in vesicular trafficking (Brodsky, 1988). Clathrin mediates budding in various membrane systems. A clathrin-PI (4,5) P2-centered pathway regulates ALR. This protein is present on autolysosomes, with exclusive enrichment on buds. Clathrin itself cannot directly anchor to membranes; instead, various adapter proteins (AP2) link clathrin to membranes. Additional studies are needed to fully understand the terminal stage of autophagy, and how this process ends in the reformation of free lysosomes.

AUTOPHAGIC PROTEINS

Cells assure the renewal of their constituent proteins through a continuous process of synthesis and degradation that also allows for rapid modulation of the levels of specific proteins to accommodate the changing extracellular environment. Intracellular protein degradation is also essential for cellular quality control to eliminate damaged or altered proteins, thus preventing the toxicity associated with their accumulation inside cells.

Autophagy essential proteins are the molecular basis of protective or destructive autophagy machinery. Some information is available regarding the signaling mechanisms governing these proteins and the opposing consequences of autophagy in mammals. Genes responsible for the synthesis of these proteins are summarized here.

Autophagy was first genetically defined in yeast, where 31 genes, referred to as autophagy-related genes (ATGs), were identified as being directly involved in the execution of autophagy (Mizushima, 2007; Xie and Klionsky, 2007). At least 16 members of this gene family have been identified in humans. The role of a large number of these genes has been deciphered. Our understanding of the molecular regulation of autophagy process originates from the characterization of these genes and proteins in yeast, many of which have counterparts in mammals. The core autophagic machinery comprises 18 Atg proteins, which represent three functional and structural units: (1) the Atg9 cycling system (Atg9, Atg1 kinase complex [Atg1 and Atg13], Atg2, Atg18, and Atg27); (2) the phosphatidylinositol 3-kinase (PI3K) complex (Atg6/Vps30), Atg14, Vps15, and Vps34; and (3) the ubiquitin-like protein system (Atg3–5, Atg7, Atg8, Atg10, Atg12, and Atg16) (Minibayeva *et al*., 2012). In addition to these core Atg proteins, 16 other proteins are essential for certain pathways or in different species.

An alternate abbreviated system of Atg proteins follows. Autophagic proteins generally function in four major groups: the Atg1 kinase complex, the VPS34 class III phosphatidylinositol 3-kinase complex, two ubiquitin-like conjugation systems involving Atg8 and Atg12, and a membrane-trafficking complex involving Atg9 (Florey and Overholtzer, 2012). In mammalian cells, the key upstream kinase that regulates the induction of most forms of autophagy is the Atg1 homologue ULK1, which forms a complex with Atg13, Fip200, and Atg101. Among the Atg proteins, Atg9 is the only multispanning membrane protein essential for autophagosome formation.

It needs to be noted that autophagy proteins are also involved in non-autophagic functions such as cell survival, apoptosis, modulation of cellular traffic, protein secretion,

cell signaling, transcription, translation, and membrane reorganization (Subramani and Malhotra, 2013). This subject is discussed in detail later in this chapter.

Protein Degradation Systems

There are two major protein degradation pathways in eukaryotic cells: the ubiquitin–proteasome system and the autophagy–lysosome system. Both of these systems are characterized by selective degradation. The ubiquitin–proteasome system (UPS) is responsible for degradation of short-lived proteins, and is involved in the regulation of various cellular signaling pathways. Autophagy is a regulatory mechanism for degrading large proteins with longer half-lives, aggregates, and defective cellular organelles. Ubiquitin binding proteins such as p62 and NBR1 regulate autophagy dynamics. These adaptor proteins decide the fate of protein degradation through either UPS or the autophagy–lysosome pathway. Many degenerative conditions, such as Huntington's, Parkinson's, Alzheimer's, amyotrophic lateral sclerosis, and diabetes, are due to defective clearance of mutated protein aggregates or defective organelles through autophagy.

Beclin 1

Beclin 1 (from Bcl-2 interacting protein) is a 60-kDa coiled-coil protein that contains a Bcl-2 homology-3 domain, a central coiled-coil domain, and an evolutionary conserved domain. Beclin 1 was originally discovered not as an autophagy protein but as an interaction partner for the anti-apoptotic protein Bcl-2. The function of Beclin 1 in autophagy was first suspected due to its 24.4% amino acid sequence identity with the yeast autophagy protein Atg6. Beclin 1 was found to restore autophagic activity in Atg6-disrupted yeast, becoming one of the first identified mammalian genes to positively regulate autophagy. Subsequent studies demonstrated that Beclin 1 is a haploinsufficient tumor-suppressor gene that is either monoallelically deleted or shows reduced expression in several different cancers (Yue *et al*., 2003).

Beclin 1 is also involved in several other biological functions, and in human conditions including heart disease, pathogen infections, impact on development, and neurodegeneration. These functions will not be discussed in this chapter because only the role of this gene (protein) in autophagy is relevant here. The central role of Beclin 1 complexes is in controlling human VPS34-mediated vesicle trafficking pathways including autophagy. Beclin 1 and its binding partners control cellular VPS34 lipid kinase activity that is essential for autophagy and other membrane trafficking processes, targeting different steps of the autophagic process such as autophagosome biogenesis and maturation (Funderburk *et al*., 2010). Beclin 1-depleted cells cannot induce autophagosome formation. In conclusion, the crucial regulator of autophagy is Beclin 1 (the mammalian homologue of yeast Atg6), which forms a multiprotein complex with other molecules such as UVRAG, AMBRA-1, Atg14L, Bif-1, Rubicon, SLAM, IP3, PINK, and survivin; this complex activates the class III phosphatidylinositol-3-kinase (Petiot *et al*., 2000).

Non-Autophagic Functions of Autophagy-Related Proteins

The importance of non-autophagic biological functions of autophagy-related proteins is beginning to be realized. These proteins (e.g., ubiquitin-like proteins Atg8 and Atg12) play

an important role in various aspects of cellular physiology, including protein sorting, DNA repair, gene regulation, protein retrotranslation, apoptosis, and immune response (Ding *et al*., 2011). They also play a role in cell survival, modulation of cellular traffic, protein secretion, cell signaling, transcription, translation, and membrane reorganization (Subramani and Malhotra, 2013). Apparently, these proteins and their conjugates possess a different, broader role that exceeds autophagy.

The interactions of ubiquitin-like proteins with other autophagy-related proteins and other proteins are summarized below. For example, six Atg8 orthologues in humans interact with at least 67 other proteins. Non-autophagy-related proteins that interact with Atg8 and LC3 include GTPases, and affect cytoskeletal dynamics, cell cycle progression, cell polarity, gene expression, cell migration, and cell transformation (Ding *et al*., 2011). Non-lipidated LC3 and non-lipidated Atg8 regulate viral replication and yeast vacuole fusion, respectively (Tamura *et al*., 2010). Atg5 and Atg12–Atg5 conjugates suppress innate antiviral immune signaling. Based on these and other functions, ubiquitin-like proteins in their conjugated and unconjugated forms modulate many cellular pathways, in addition to their traditional role in autophagy (Subramani and Malhotra, 2013).

In addition to ubiquitin-like Atg proteins, other Atg-related proteins are also involved in non-autophagic functions; these are summarized below. UNC-51, the homologue of human ULK1, regulates axon guidance in many neurons. Atg16L1 positively modulates hormone secretion in PC12 cells, independently of autophagic activity (Ishibashi *et al*., 2012). Atg16L1, Atg5, Atg7, and LC3 are genetically linked to susceptibility to Crohn's disease, a chronic inflammatory condition of the intestinal tract (Cadwell *et al*., 2009). Atg5, Atg7, Atg4B, and LC3 are involved in the polarized secretion of lysosomal enzymes into an extracellular resorptive space, resulting in the normal formation of bone pits or cavities (bone resorption) (Deselm *et al*., 2011).

The wide variety of functions of Atg-related proteins in typical non-autophagic cellular activities (some of which are enumerated here) indicates that the autophagic machinery is enormously complex and more versatile than presently acknowledged. Indeed, much more effort is needed to better understand the role of this machinery in health and disease, which eventually may allow us to delay the aging process and provide us with effective therapeutics.

Microtubule-Associated Protein Light Chain 3

Microtubule-associated protein chain 3 (LC3) is a mammalian homologue of yeast Atg8. It was the first mammalian protein discovered to be specifically associated with autophagosomal membranes. Although LC3 has a number of homologues in mammals, LC3B is most commonly used for autophagy (macroautophagy) assays because it plays an indispensable role in autophagy formation, making it a suitable marker for the process.

The cytoplasm contains not only LC3-I but also an active form (LC3-II). Immediately after synthesis of the precursor protein (pro-LC3), hAtg4B cleaves a C-terminal 22-amino acid fragment from this precursor form to the cytosolic form LC3-I. Afterwards, LC3-I is transiently conjugated to membrane-bound phosphatidylethanolamine (PE) to generate LC3-II, which localizes in both the cytosolic and intralumenal faces of autophagosomes. Because of its essential role in the expansion step of autophagosome formation, LC3-II is regarded as the most reliable marker protein for autophagy. Following fusion with lysosomes,

intralumenally-located LC3-II is degraded by lysosomal hydrolases, and cytosolically-oriented LC3-II is delipidated by hAtg4B, released from the membrane, and finally recycled back to LC3-I (Karim *et al.*, 2007). Divergent roles of LC3 (or Beclin 1) in tumorigenesis have been reported. For example, LC3 expression is either decreased in brain cancer (Aoki *et al.*, 2008) and ovary cancer (Shen *et al.*, 2008) or increased in esophageal and gastrointestinal neoplasms (Yoshioka *et al.*, 2008). LC3 is also associated with a poor outcome in pancreatic cancer (Fujita *et al.*, 2008), whereas its expression is associated with a better survival in glioblastoma patients with a poor performance score (Aoki *et al.*, 2008). It has also been reported that LC3-II protein expression is inversely correlated with melanoma thickness, ulceration, and mitotic rate (Miracco *et al.*, 2010). These and other studies imply that the clinical impact of LC3 is associated with the tumor type, tissue context, and other factors.

MONITORING AUTOPHAGY

A number of methods are available to monitor autophagy; such monitoring can be accomplished by using electron microscopy, biochemical protocols, and detection of relevant protein modifications through SDS-PAGE and western blotting. Autophagy can be monitored by detecting autophagosomal proteins such as LC3. LC3 is a specific marker protein of autophagic structure in mammalian cultured cells. The appearance of this protein-positive puncta is indicative of the induction of autophagy. One such method consists of monitoring autophagy by detecting LC3 conversion from LC3-I to LC3-II by immunoblot analysis because the amount of LC3-II is clearly correlated with the number of autophagosomes. Endogenous LC3 is detected as two bands following SDS-PAGE and immunoblotting: one represents cytosolic LC3-I and the other LC3-II that is conjugated with phosphatidylethanolamine, which is present on isolation membranes and autophagosomes but much less so on autolysosomes (Mizushima and Yoshimori, 2007). According to Kadowaki and Karim (2009), the LC3-I to LC3-II ratio in the cytosol (cytosolic LC3 ratio), but not in the homogenate, is an easy quantitative method for monitoring the regulation of autophagy. Alternatively, comparison of LC3-II levels between different conditions is a useful method for monitoring autophagy.

Another approach is use of the fluorescent protein GFP-LC3, which is a simple and specific marker. To analyze autophagy in whole animals, GFP-LC3 transgenic mice have been generated (Mizushima and Kuma, 2008). However, the GFP-LC3 method does not provide a convenient measure for assessing autophagic flux. Therefore, another alternative method, tandem fluorescent-tagged LC# (tfLC#) can be used to monitor autophagic flux (Kimura *et al.*, 2009).

In spite of the advantages of the LC3 method, it has some limitations. LC3 protein, for example, tends to aggregate in an autophagy-independent manner. LC3-positive dots seen in the light microscope after using the transfected GFP-LC3 method may represent protein aggregates, especially when GFP-LC3 is overexpressed or when aggregates are found within cells (Kuma *et al.*, 2007). LC3, in addition, is easily incorporated into intracellular protein aggregates – for example, in autophagy-deficient hepatocytes, neurons, or senescent fibroblasts. Also, LC3 is degraded by autophagy.

In light of the above limitations, it is important to measure the amount of LC3-II delivered to lysosomes by comparing its levels in the presence of or absence of lysosomal protease inhibitors such as E64d and pepstatin A (Mizushima and Yoshimori, 2007). These authors have pointed out pitfalls and necessary precautions regarding LC3 immunoblot analysis. A very extensive update of the assays for monitoring autophagy has been presented by Klionsky *et al.* (2012), who strongly recommend the use of multiple assays to monitor autophagy, and present 17 methods of doing so.

REACTIVE OXYGEN SPECIES (ROS)

Reactive oxygen species (ROS) are highly reactive forms of molecular oxygen, including the superoxide anion radical, hydrogen peroxide, singlet oxygen, and hydroxyl radical (Park *et al.*, 2012). ROS are generally produced during normal metabolism of oxygen inside the mitochondrial matrix that acts as the primary source of them. Basal levels of ROS serve as physiological regulators of normal cell multiplication and differentiation. If the balance of ROS increases more than the scavenging capacity of the intracellular antioxidant system, the cell undergoes a state of oxidative stress with significant impairment of cellular structures. Excessive levels of ROS, for example, can cause severe damage to DNA and proteins.

The oxidative stress especially targets mitochondria, resulting in the loss of mitochondrial membrane potential and initiating mitochondria-mediated apoptosis. Oxidative stress can also lead to the auto-oxidation of sterols, thereby affecting the cholesterol biosynthetic pathway – mainly the postlanosterol derivatives. The intracellular accumulation of oxysterols directs the cell to its autophagic fate, and may also induce it to differentiate. ROS, in fact, can play contrasting roles: they can initiate autophagic cell death and also function as a survival mechanism through induction of cytoprotective autophagy in several types of cancer cells.

MAMMALIAN TARGET OF RAPAMYCIN (mTOR)

The mammalian target of rapamycin (mTOR), also known as the mechanistic target of rapamycin or FK506-binding protein 12-rapamycin-associated protein 1 (FRAP1), is an ~289-kDa protein originally discovered and cloned from *Saccharomyces cerevisiae* that shares sequence homologues with the phosphoinositide 3-kinase (PI3-kinase) family, which is the key element in response to growth factors. mTOR represents a serine threonine protein kinase that is present in all eukaryotic organisms (Wullschleger *et al.*, 2006). mTOR represents the catalytic subunit of two distinct complexes, mTORC1 and mTORC2 (Zoncu *et al.*, 2011). mTORC1 controls cell growth by maintaining a balance between anabolic processes (e.g., macromolecular synthesis and nutrient storage) and catabolic processes (e.g., autophagy and the utilization of energy stores) (Nicoletti *et al.*, 2011). The receptor–mTOR complex positively regulates cell growth, and its inhibition causes a significant decrease in cell size. The raptor part of the mTOR pathway modulates a large number of major processes that are listed here.

Rapamycin binds to the FKBP12 protein, forming a drug–receptor complex which then interacts with and perturbs TOR. TOR is the central component of a complex signaling network that regulates cell growth and proliferation. The components of these complexes exist in all eukaryotes.

As indicated above, mTOR is a major cellular signaling hub that integrates inputs from upstream signaling pathways, including tyrosine kinase receptors, that play a key role in intracellular nutrient sensoring. It serves as the convergent point for many of the upstream stimuli to regulate cell growth and nutrient metabolism, cell proliferation, cell motility, cell survival, ribosome biosynthesis, protein synthesis, mRNA translation, and autophagy (Meijer and Godogno, 2004). Two mammalian proteins, S6 kinase and 4E-BP1, link raptor–mTOR to the control of mRNA translation (Sarbassov *et al*., 2005).

mTOR also governs energy homeostasis and cellular responses to stress, such as nutrient deprivation and hypoxia. Many studies have demonstrated that the Akt/mTOR-dependent pathway is involved in the process of chemical (platinum)-induced autophagy, in which mTOR is a pivotal molecule in controlling autophagy by activating mTOR (Hu *et al*., 2012). Another recent investigation also shows that methamphetamine causes damage to PC12 cells, but this damage can be decreased by using a supplement of taurine via inhibition of autophagy, oxidative stress, and apoptosis (Li *et al*., 2012).

Abundance of nutrients, including growth factors, glucose, and amino acids, activates mTOR and suppresses autophagy, while nutrient deprivation suppresses mTOR, resulting in autophagy activation. In other words, triggering of autophagy relies on the inhibition of mammalian mTOR, an event that promotes the activation of several autophagy proteins (Atgs) involved in the initial phase of membrane isolation. Among many signaling pathways controlling mTOR activation, phosphoinositide 3-kinase (PI3K) is the key element in response to growth factors. mTORC1 and Atg1–ULK complexes constitute the central axis of the pathways that coordinately regulate growth and autophagy in response to cellular physiological and nutritional conditions. The negative regulation of mTORC1 by Atg1–ULK stresses further the intimate cross-talk between autophagy and cell growth pathways (Jung *et al*., 2010).

ROLE OF AUTOPHAGY IN TUMORIGENESIS AND CANCER

Malignant neoplasms constitute the second most common cause of death in the United States, and malignant brain tumors contribute 2.4% of cancer-related deaths. An estimated 20,340 new cases of primary central nervous system tumors were diagnosed in 2012 in the United States alone, and resulted in approximately 13,110 deaths. Despite considerable advances in multimodal treatment of tumors in the past five decades, there has been only a minimal improvement in the median survival time of brain-malignancy patients. Causative factors for the poor survival rate include the highly invasive nature of brain malignant tumors, making them intractable to complete surgical resection, and resistance to standard chemotherapy and radiotherapy. This difficulty in remedying cancer underscores the need to pursue prosurvival signaling mechanisms that contribute to the resistance of cancer development; such alternative therapies include the use of autophagy.

Autophagy defects are linked to many diseases, including cancer, and its role in tumorigenesis, being tissue- and genetic context-dependent, is exceedingly complex. Metabolically

stressed tumor cells rely on autophagy for survival and reprogramming of their metabolism to accommodate rapid cell growth and proliferation (Lozy and Karantza, 2012). To accomplish this goal, specific catabolic reactions (e.g., aerobic glycolysis and glutaminolysis) are upregulated to provide needed energy and rebuild new complex macromolecules such as proteins, nucleic acids, and lipids.

Autophagy has complex and paradoxical roles in antitumorigenesis, tumor progression, and cancer therapeutics. Initially, two principal lines of evidence connected autophagy and cancer: it was found that (1) the *BECN1* gene is monoallelically deleted in several types of cancers, and (2) autophagy can function to promote tumor cell survival, but can also contribute to cell death. In other words, autophagy can be both tumorigenic and tumor suppressive. Its exact role in each case is dependent on the context and stimuli. Autophagy can be upregulated or suppressed by cancer therapeutics, and upregulation of autophagy in cancer therapies can be either prosurvival or prodeath for tumor cells.

It is known that autophagy maintains cellular integrity and genome stability. Loss of autophagy genes perturbs this homeostasis, thereby potentially priming the cell for tumor development. The following autophagy genes are frequently mutated in human cancers (Liu and Ryan, 2012): *BECN1*, *UVRAG*, *SH3GLB1* (Bif-1), *Atg2B*, *Atg5*, *Atg9B*, *Atg12*, and *RAB7A*. Mutations in *Atg2B*, *Atg5*, *Atg9B*, and *Atg12* have been reported in gastric and colorectal cancers (Kang *et al.*, 2009). The expression of Bif-1 is downregulated in gastric and prostate cancers (Takahashi *et al.*, 2010). Mutations of *UVRAG* have been found in colon cancer (Knaevelsrud *et al.*, 2010).

Autophagy is associated with both cancer progression and tumor suppression. The molecular mechanisms underlying these two phenomena have been elucidated. It is known that cancer cells generally tend to have reduced autophagy compared with their normal counterparts and premalignant lesions. Therefore, for autophagy to induce cancer progression, it will have to be activated. This is accomplished, for example, by the KRAS oncogene, which is known to induce autophagy. It has been shown that autophagy is activated constitutively in oncogenic KRAS-driven tumors, and that this cellular event is required for the development of pancreatic tumors (Yang *et al.*, 2011).

The discovery that the autophagic-related gene *BECN 1* suppresses tumor growth stimulated significant interest from cancer biologists in this previously unexplored therapeutic process. This interest has resulted in both intensive and extensive research efforts to understand the role of autophagy in cancer initiation, progression, and suppression. Pharmacological or genetic inactivation of autophagy impairs KRAS-mediated tumorigenesis. It has been shown that transmembrane protein VMP1 (vacuole membrane protein 1), a key mediator of autophagy, is a transcriptional target of KRAS signaling in cancer cells (Lo Ré *et al.*, 2012). It regulates early steps of the autophagic pathway. In fact, KRAS requires VMP1 not only to induce but also to maintain autophagy levels in cancer. PI3K–AKT1 is the signaling pathway mediating the expression and promoter activity of VMP1 upstream of the GLI3–p300 complex.

The *BECN 1* gene is deleted in ~40% of prostate cancers, ~50% of breast cancers, and ~75% of ovarian cancers (Liang *et al.*, 1999). In addition, reduced expression of Beclin 1 has been found in other types of cancers, including human colon cancer, brain tumors, hepatocellular carcinoma, and cervical cancer. It can be concluded that a defective autophagic process is clearly linked to cancer development.

Autophagy is associated with resistance to chemotherapeutics such as 5-fluorouracil and cisplatin. It is recognized that tumors and the immune systems are intertwined in a competition where tilting the critical balance between tumor-specific immunity and tolerance can finally determine the fate of the host (Townsend *et al.*, 2012). It is also recognized that defensive and suppressive immunological responses to cancer are exquisitely sensitive to metabolic features of rapidly growing tumors.

On the other hand, autophagy may increase the effectiveness of anticancer radiotherapy. It is known that some malignancies become relatively resistant to repeated radiotherapy, and may eventually recover self-proliferative capacity. This problem can be diminished by inducing autophagy through Beclin 1 overexpression in conjunction with radiotherapy. It is known that autophagy enhances the radiosensitization of cancer cells rather than protecting them from radiation injury and cell death. It is also known that autophagy inhibits the growth of angiogenesis in cancer cells. It should also be noted that autophagic cell death occurs in many cancer types in response to various anticancer drugs. In other words, autophagy can serve as a pathway for cellular death. Based on the two opposing roles of autophagy, it is poised at the intersection of life and death. It is apparent that we need to understand and modulate the autophagy pathway to maximize the full potential of cancer therapies.

As mentioned earlier, autophagy is frequently upregulated in cancer cells following standard treatments (chemotherapy, radiotherapy), showing as prosurvival or prodeath for cancer cells (reviewed by Liu and Ryan, 2012). Treatment with rapamycin, rapamycin analogues, and imatinib shows a prodeath effect, while treatment with radiation, tamoxifen, camptothecan, and proteasome inhibitors results in the survival of cancer cells. The effect of autophagy seems to be different in distinct tumor types, at various stages of tumor development, and even within different regions of the same tumor. It is concluded that, generally, either overactivation or underactivation of autophagy contributes to tumorigenesis, and that autophagy limits tumor initiation, but promotes establishment and progression.

ROLE OF AUTOPHAGY IN IMMUNITY

The eradication of invading pathogens is essential in multicellular organisms, including humans. During the past two decades there has been rapid progress in the understanding of the innate immune recognition of microbial components and its critical role in host defense against infection. The innate immune system is responsible for the initial task of recognizing and destroying potentially dangerous pathogens. Innate immune cells display broad antimicrobial functions that are activated rapidly upon encountering microorganisms (Franchi *et al.*, 2009).

Autophagy can function as a cell's defense against intracellular pathogens. It is involved in almost every key step, from the recognition of a pathogen to its destruction and the development of a specific adaptive immune response to it. Autophagy, in addition, controls cell homeostasis and modulates the activation of many immune cells, including macrophages, dendritic cells, and lymphocytes, where it performs specific functions such as pathogen killing or antigen processing and presentation (Valdor and Macian, 2012).

The autophagy pathway is linked to one or more aspects of immunity. Studies have shown that autophagy is regulated by pathways that are critical for the function and differentiation of cells of the immune system, including Toll-like receptors (TLRs). TLRs were the first class of immune receptors identified as regulators in cells of the innate immune system, and play a crucial role in many aspects of the immune response. They are broadly expressed in immune cells, particularly in antigen-presenting cells, and recognize pathogen-associated molecular patterns such as lipopolysaccharides, viral double-stranded RNA, and unmethylated CPG islands (Harashima *et al*., 2012). Initiation of TLR signaling induces release of inflammatory cytokines, maturation of dendritic cells, and activation of adaptive immunity. Cancer cells also express functional TLRs. TLR4 signaling, for example, promotes escape of human lung cancer cells from the immune system by inducing immune suppressive cytokines and promoting resistance to apoptosis (He *et al*., 2007). In contrast, TRL3 signaling induces antitumor effects. Akt activation can render cancer cells resistant to antitumor cellular immunity (Hähnel *et al*., 2008). The implication is that Akt inactivation increases the susceptibility of cancer cells to immune surveillance.

TLRs also have been shown to induce autophagy in several cell types, including neutrophils (Xu *et al*., 2007). Activation of the TLR downstream signaling proteins MyD88 and Trif appears to be involved in the induction of autophagy. These proteins are recruited together with Beclin 1 to TLR4, which promotes the dissociation of the Beclin 1–Bc12 complex and induces autophagosome formation (Shi and Kehri, 2008). MyD88 and Trif target Beclin 1 to trigger autophagy in macrophages. TLRs have also been shown to promote a process involving the autophagy machinery termed LC3-associated phagocytosis (Valdor and Macian, 2012). The uptake of cargo containing TLR ligands by macrophages leads to the recruitment of LC3 on the phagosome surface, promoting degradation of the pathogens by enhancing phagosome–lysosome fusion in the absence of autophagosome formation (Sanjuan *et al*., 2009).

In fact, the study of TLRs showed that pathogen recognition by the innate immune system is specific, relying on germline-encoded pattern-recognition receptors that have evolved to detect components of foreign pathogens (Akira *et al*., 2006). TLRs recognize conserved structures in pathogens, which leads to the understanding of how the body senses pathogen invasion, triggers innate immune responses, and primes antigen-specific adaptive immunity (Kawai and Akira, 2010). The adaptive immune system relies on a diverse and specific repertoire of clonally selected lymphocytes. Additional studies are needed to better understand the mechanisms that regulate autophagy in immune cells and the role this process plays in the establishment of immune responses against foreign pathogens.

AUTOPHAGY AND SENESCENCE

Cellular senescence is a biological state in which cells have lost the ability of undergoing mitosis, but remain metabolically active for a long time. Three types of senescence have been reported:

1. Replicative senescence, caused by telomere shortening after a genetically predetermined number of cell divisions in non-transformed cells (Shay and Roninson, 2004).

2. Oncogene-induced senescence, which involves the capacity of cells to undergo senescence in the presence of oncogenes (e.g., Ras) (Lee *et al.*, 1999).
3. Premature senescence, occurring through exposure of cells to exogenous cytotoxic agents causing DNA damage (Gewirtz, 2014).

It is known that the cytotoxic response of autophagy to stress and stress-induced senescence evades cell death. However, autophagy can be either a cytoprotective or cytotoxic response to chemotherapy or radiotherapy. Some information is available regarding a relationship between autophagy and senescence. That there is a cross talk between autophagy and apoptosis has also been established, and this is discussed elsewhere in this chapter.

An increase of autophagic vacuoles and senescence has been observed in the bile duct cells of patients with primary biliary cirrhosis (Sasaki *et al.*, 2010). The generation of autophagic vesicles in dying senescent keratinocytes has also been reported (Gosselin *et al.*, 2009), and Patschan and Goligorsky (2008) have found autophagy markers in senescent endothelial cells. More importantly, Young *et al.* (2009) reported the upregulation of autophagy-related genes during oncogene-induced senescence, and that inhibition of autophagy delayed the senescence phenotype. Recently, Goehe *et al.* (2012) reported that treatment of breast cancer cells and colon cancer cells with doxorubicin or camptothecin resulted in both autophagy and senescence.

It is concluded that both autophagy and senescence are collaterally induced by chemotherapy in cancer cells. In contrast, interference with ROS generation, ATM activation, and induction of p53 or p21 suppresses both autophagy and senescence (Goehe *et al.*, 2012). Both autophagy and senescence signal to the immune system the presence of tumor cells that require elimination. In addition, both autophagy and senescence enhance the effect of chemotherapy on cancer cells. Although autophagy accelerates the senescence process by possibly providing an additional source of energy, senescence can occur independently of autophagy.

ROLE OF AUTOPHAGY IN VIRAL DEFENSE AND REPLICATION

Viruses and other pathogens induce dramatic changes in the intracellular environment. Infected cells activate certain defense pathways to combat these pathogens. Conversely, pathogens interfere with defense processes and utilize cellular supplies for pathogen propagation. Autophagy, for example, plays an antiviral role against the mammalian vesicular stomatitis virus, and the phosphatidylinositol 3-kinase–Akt signaling pathway is involved in this defense process (Shelly *et al.*, 2009). Many virus types, including herpes simplex virus 1 and Sindbis virus, have been observed inside autophagic compartments for degradation (Orvedahl *et al.*, 2007).

Autophagy is an essential component of *Drosophila* immunity against the vesicular stomatitis virus (Shelly *et al.*, 2009). Recently, an interesting role of the RNAse L system and autophagy in the suppression or replication of the encephalomyocarditis virus or vesicular stomatitis virus was reported (Chakrabarti *et al.*, 2012). At a low multiplicity of infection, induction of autophagy by RNAse L suppresses virus replication; however, in subsequent rounds of infection, autophagy promotes viral replication. RNAse is a virus-activated host RNAse pathway that disposes of or processes viral and cellular single-stranded RNAs.

However, it has not been established whether autophagy itself is sufficient to control viral replication in all cases; the participation of other cell death phenomena in this defense process cannot be disregarded. On the other hand, autophagy is, for example, actively involved in influenza A virus replication (Zhou *et al*., 2009). Mouse hepatitis virus and polio virus sabotage the components of the mammalian autophagy system, which normally is important in innate immune defense against intracellular pathogens. In other words, autophagic machinery (which normally would function to eliminate a virus) may promote viral assembly (Jackson *et al*., 2005). However, Zhao *et al*. (2007) indicate that mouse hepatitis virus replication does not require the autophagy gene *Atg5*.

The survival of HIV depends on its ability to exploit the host cell machinery for replication and dissemination, to circumvent the cell's defense mechanisms or to use them for its replication. Autophagy plays a dual role in HIV-1 infection and disease progression. Direct effects of HIV on autophagy include the subversion of autophagy in HIV-infected cells and the induction of hyper-autophagy in bystander CD4+ T cells. HIV proteins modulate autophagy to maximize virus production (Killian, 2012). On the other hand, HIV-1 protein also disrupts autophagy in uninfected cells and thus contributes to CD4+ T cell death and viral pathogenesis.

It has also been reported that HIV-1 downregulates autophagy regulatory factors, reducing both basal autophagy and the number of autophagosomes per cell (Blanchet *et al*., 2010). The HIV negative elongation factor (Nef) protein protects HIV from degradation by inhibiting autophagosome maturation (Kyei *et al*., 2009). It has been shown that the foot and mouth disease virus induces autophagosomes during cell entry to facilitate infection, but does not provide membranes for replication (Berrym *et al*., 2012).

Another example of a virus that uses a component of autophagy to replicate itself is the hepatitis C virus (HCV) (Sir *et al*., 2012). HCV perturbs the autophagic pathway to induce the accumulation of autophagosomes in cells (via the PI3KC3-independent pathway) and uses autophagosomal membranes for its RNA replication. Other positive-strand RNA viruses (poliovirus, dengue virus, rhinoviruses, and nidoviruses) also use the membrane of autophagic vacuoles for their RNA replication (Sir and Ou, 2010). Suppression of LC3 and Atg7 reduces the HCV RNA replication level; these two proteins are critical for autophagosome formation. There is still controversy regarding the contrasting roles of autophagy in pathogen invasion; the mechanisms governing activation of autophagy in response to virus infection require further elucidation.

ROLE OF AUTOPHAGY IN INTRACELLULAR BACTERIAL INFECTION

Post-translation modifications of cell proteins (e.g., ubiquitination) regulate the intracellular traffic of pathogens. Ubiquitination involves the addition of ubiquitin to the lysine residues of target proteins, resulting in endocytosis and sorting events (Railborg and Stenmark, 2009). Several strategies have been developed by pathogenic bacteria to interfere with the host's ubiquitination and thus to achieve successful infection. Some types of bacteria act directly on the ubiquitination pathway by mimicking host cell proteins, while others (e.g.,

Escherichia coli, *Shigella flexneri*) act indirectly by expressing or interfering with the host ubiquitinating pathway. The other defense by the cell against bacterial infection is through autophagy; this is described below.

Autophagy serves as a double-edged sword; on the one hand it eliminates some pathogens and bacterial toxins, while on the other hand some pathogens can evade or exploit autophagy for survival and replication in a host. Recently, it has become clear that the interaction between autophagy and intracellular pathogens is highly complex. The components of the autophagy machinery also play roles in infection in a process different from the canonical autophagy pathway (formation of a double-membrane autophagosome and the involvement of more than 35 autophagy-related proteins, including the LC3 mammalian autophagy marker). There is an alternative autophagy pathway that is relevant to infection. For example, a subset of autophagy components can lead to LC3 conjugation onto phagosomes (Cemma and Brumell, 2012). In other words, the process of LC3-associated phagocytosis (LAP) results in the degradation of the cargo by promoting phagosome fusion with lysosomes. It is likely that both the LAP process and the canonical system operate simultaneously or selectively as host defenses against infection. Examples of bacteria the growth of which is suppressed by autophagy include *Escherichia coli* (Cooney *et al.*, 2010), *Salmonella typhimurium* (Perrin *et al.*, 2004), *Streptococcus pyogenes* (Virgin and Levine, 2009), and *Mycobacterium tuberculosis* (Randow, 2011); examples of bacteria that exploit autophagy for replication include *Staphylococcus aureus*, *Legionella pneumophila*, and *Yersinia pseudotuberculosis*; examples of bacteria that can evade targeting by autophagy/LAP include *Listeria monocytogenes* (Randow, 2011), *Shigella flexneri* (Virgin and Levine, 2009), and *Burkholderia pseudomallei*.

ROLE OF AUTOPHAGY IN HEART DISEASE

Heart failure is one of the leading causes of morbidity and mortality in industrialized countries. Myocardial stress due to injury, valvular heart disease, or prolonged hypertension induces pathological hypertrophy, which contributes to the development of heart failure and sudden cardiac death (Ucar *et al.*, 2012).

It has been reported that autophagy is an adaptive mechanism to protect the heart from hemodynamic stress. In fact, autophagy plays a crucial role in the maintenance of cardiac geometry and contractile function (Nemchenko *et al.*, 2011). Cardiac-specific loss of autophagy causes cardiomyopathy. Impaired autophagy has been found in a number of heart diseases, including ischemia/reperfusion injury. Excessive and uncontrolled autophagy leads to loss of functional proteins, depletion of essential organic molecules, oxidative stress, loss of ATP, the collapse of cellular catabolic machinery, and, ultimately, the death of cells in the heart. Autophagic elimination of damaged organelles, especially mitochondria, is crucial for proper heart function, whereas exaggerated autophagic activity may foster heart failure. Therefore, a delicate balance of autophagy maintains cardiac homeostasis, whereas an imbalance leads to the progression of heart failure.

A consensus on whether autophagy is cardioprotective or leads to hypertrophy and heart failure is lacking. In any case, autophagy is an important process in the heart. Various studies indicate that autophagy has a dual role in the heart, where it can protect against or contribute to cell death depending on the stimulus. It occurs at low basal levels under normal

conditions, and is important for the turnover of organelles. Autophagy is upregulated in the heart in response to stress such as ischemia/reperfusion. Studies of ischemia/reperfusion injury indicate that ROS and mitochondria are critical targets of injury, as opening of the mitochondrial permeability transition pore culminates in cell death. However, Sciarretta *et al.* (2011) indicate that autophagy is beneficial during ischemia but harmful during reperfusion.

It has been shown that mitophagy mediated by Parkin is essential for cardioprotection (Huang *et al.*, 2011). The sequestration of damaged mitochondria depends on Parkin, which averts the propagation of ROS-induced ROS release and cell death. The implication is that mitochondrial depolarization and removal through mitophagy is cardioprotective. The sequestration of damaged cell materials into autophagosomes is essential for cardioprotection. An increased number of autophagosomes is a prominent feature in many cardiovascular diseases, such as cardiac hypertrophy and heart failure (Zhu *et al.*, 2007). Recently, Gottlieb and Mentzer (2013) have ably reconciled contradictory findings and concluded that the preponderance of evidence leans towards a beneficial role of autophagy in the heart under most conditions.

Recently, it was reported that autophagy plays a role in the onset and progression of alcoholic cardiopathy (Guo and Ren, 2012). Adenosine monophosphate-activated protein kinase (AMPK) plays a role in autophagic regulation and subsequent changes in cardiac function following an alcoholic challenge. It is known that AMPK promotes autophagy via inhibition of mTORC1 by phosphorylating the mTORC1-associated protein Raptor and tuberous sclerosis complex 2.

MicroRNAs (miRNAs) also play a role in cardiomyopathy and heart failure. These endogenous small molecules regulate their target gene expression by post-transcriptional regulation of messenger RNA. Recently, it was demonstrated that hypertrophic conditions induced the expression of the miR-212/132 family in cardiomyocytes, and both of these molecules regulated cardiac hypertrophy and cardiomyocyte autophagy (Ucar *et al.*, 2012). Cardiac hypertrophy and heart failure in mice can be rescued by using a pharmacological inhibitor of miR-132.

Inflammation is also implicated in the pathogenesis of heart failure. Some information is available regarding the mechanism responsible for initiating and integrating inflammatory responses within the heart. Mitochondrial DNA plays an important role in inducing and maintaining inflammation in the heart. Mitochondrial DNA that escapes from autophagy cells autonomously leads to Toll-like receptor (TLR) 9-mediated inflammatory responses in cardiomyocytes, and is capable of inducing myocarditis and dilated cardiomyopathy (Oka *et al.*, 2012). Pressure overload induces the impairment of mitochondrial cristae morphology and functions in the heart. It is known that mitochondria damaged by external hemodynamic stress are degraded by the autophagy/lysosome system in cardiomyocytes (Nakai *et al.*, 2007). It is also known that increased levels of circulating proinflammatory cytokines are associated with disease progression and adverse outcomes in patients with chronic heart failure.

ROLE OF AUTOPHAGY IN NEURODEGENERATIVE DISEASES

Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD) are the major neurodegenerative conditions causing dementia and movement disorders in the aging population. All three diseases are characterized by the presence of abnormal protein aggregates and neuronal death, although the etiology of AD is distinct from that of PD and HD.

It is known that epigenetic dysregulation and transcriptional dysregulation are pathological mechanisms underlying neurological diseases. It is also known that histone deacetylase (HDAC) inhibitor 4b preferentially targets HDAC1 and HDAC3, ameliorating, for example, HD (Jia *et al*., 2012). HDACs are enzymes that remove acetyl groups from lysine amino acid on a histone. Several studies have identified HDAC inhibitors (4b) as candidate drugs for the treatment of neurodegenerative diseases, including HD.

Familial AD mutations increase the amyloidogenicity of the amyloid beta peptide, placing disruption of amyloid precursor protein (APP) metabolism and amyloid beta production at the center of AD pathogenesis (Pickford *et al*., 2008). An increase in the production of both APP and amyloid beta, and a decrease in the degradation of APP, contributes to AD.

PD is a progressive neurodegenerative disorder caused by the interaction of genetic and environmental factors. It is characterized by the loss of dopaminergic neurons. The available evidence indicates that mitochondrial dysfunction, environmental toxins, oxidative stress, and abnormal accumulation of cytoplasmic proteinaceous materials can contribute to disease pathogenesis. These proteins tend to aggregate within Lewy bodies. The loss of dopaminergic neurons in the substantia nigra may be partly due to the accumulation of aggregated or misfolded proteins or mitochondrial dysfunction. Prevention of such accumulation or degeneration of dysfunctional mitochondria might prevent the occurrence of apoptosis. Mutations in the DJ-1 oncogene are also implicated in the pathogenesis of this disease. This oncogene is neuroprotective by activating the ERK1/2 pathway and suppressing mTOR in the dopaminergic neurons, leading to enhanced autophagy.

One of the major constituents of Lewy bodies is a protein called alpha-synuclein. This protein is likely to be a toxic mediator of pathology in PD because wild-type alpha-synuclein gene duplications, which increase its expression levels, cause rare cases of autosomal dominant PD (Winslow and Rubinsztein, 2011). Overexpression of alpha-synuclein increases mutant huntingtin aggregation. Mutant huntingtin is an autophagy substrate, and its level increases when autophagy is compromised. Even physiological levels of this protein negatively regulate autophagy.

HD is characterized by the accumulation of mutant huntingtin (the protein product of the *IT15* gene) in intraneuronal inclusions, primarily in the brain but also peripherally. The increase is caused by the appearance of cytoplasmic (neutrophil) and nuclear aggregates of mutant huntingtin, and selective cell death in the striatum and cortex (DiFiglia *et al*., 1997). HD is recognized as a toxic gain-of-function disease, where the expansion of the polyQ stretch within huntingtin confers new deleterious functions on the protein. Loss of normal huntingtin function is thought to be responsible for HD.

Amyotrophic lateral sclerosis (ALS) is the fourth most common neurodegenerative disease. It is characterized by progressive loss of upper and motor neurons. The following genes and proteins have been reported to be involved in familial ALS: superoxide dismutase 1, als2, TAR DNA binding protein 43 kDa, and optineurin (Da Cruz and Cleveland, 2011). Accumulation of ubiquitinated inclusions containing these gene products is a common feature in most familial ALS models, and is also a pathologic hallmark of sporadic ALS. Failure to eliminate detrimental proteins is linked to pathogenesis of both familial and sporadic types of ALS. Dysfunction of 26S proteasome in motor neurons is sufficient to induce cytopathological phenotypes of ALS (Tashiro *et al*., 2012). This evidence indicates that dysfunction of the ubiquitin–proteasome system primarily contributes to the pathogenesis of sporadic ALS. In other words,

proteasomes, but not autophagy, fundamentally govern the development of ALS, in which TDP-43 and FUS proteinopathy plays a crucial role (Tashiro *et al.*, 2012). The role of autophagy in AD, PD, and HD is further elaborated below.

Loss of autophagy-related genes results in neurodegeneration and abnormal protein accumulation. Autophagy is important in avoiding, or at least delaying, the development of age-related diseases such as neurodegeneration and cancer. In fact, autophagy is an essential pathway in postmitotic cells, such as neurons, that are particularly susceptible to the accumulation of defective proteins and organelles. Neuron-specific disruption of autophagy results in neurodegenerative diseases, including AD, PD, HD, ALS, and prion diseases. Tissue-specific genetic manipulation of autophagy of the brain causes neuronal accumulation of misfolded proteins and an accelerated development of neurodegeneration.

One of the prominent features of AD is the accumulation of autophagic vacuoles in neurons, suggesting dysfunction in this degradation pathway. Autophagy is normally efficient in the brain, as reflected by the low number of brain autophagic vacuoles at any given moment (Nixon and Yang, 2011). In contrast, brains of AD patients exhibit prominent accumulation of such vacuoles in association with dystrophic neuritis and deformed synaptic membranes (Yu *et al.*, 2005).

The majority of PD is idiopathic, with no clear etiology. The available evidence indicates that mitochondrial dysfunction, environmental toxins, oxidative stress, and abnormal protein accumulation can contribute to disease pathogenesis. The loss of dopaminergic neurons in the substantial nigra may be partly due to the accumulation of aggregated or misfolded proteins, or mitochondrial dysfunction. Prevention of such accumulations or degradation of dysfunctional mitochondria might prevent the occurrence of apoptosis. Mutations in the DJ-1 oncogene are also implicated in the pathogenesis of this disease. DJ-1 is neuroprotected by activating the ERL1/2 pathway and suppressing mTOR in the dopaminergic neurons, leading to enhanced autophagy. Upregulation of autophagy has the potential to be a therapeutic strategy for disorders. This genetic method for autophagy upregulation is mTOR-independent. The development of genetic-based therapeutic strategies aimed at stimulating the autophagic clearance of aggregated proteins can be used both in the treatment of neurodegenerative diseases and in lifespan extension (Zhang *et al.*, 2010). Several studies have identified histone deacetylose (HDAC) inhibitors (4b) as candidate drugs for the treatment of neurological diseases, including HD.

CROSS-TALK BETWEEN AUTOPHAGY AND APOPTOSIS

The cross-talk between autophagy and apoptosis is exceedingly complex, and various aspects of this phenomenon are still being understood. A brief introduction to the apoptosis pathway is in order. The significant functions of apoptosis (type 1 programmed cell death) are embodied in its maintenance of organism homeostasis and metabolic balance, and organ development. Morphological changes and death in apoptotic cells are caused by caspases, which cleave 400 proteins. The earliest recognized morphological changes in apoptosis involve condensation of cytoplasm and chromatin, DNA fragmentation, and cell shrinkage. The plasma membrane convolutes or blebs in a florid manner, producing fragments of a cell (apoptotic bodies). The fragments are membrane bound, and contain

nuclear parts. The apoptotic bodies are rapidly taken up by nearby cells and degraded within their lysosomes.

There are two established signaling pathways that result in apoptosis. In the extrinsic pathway, apoptosis is mediated by death receptors on the cell surface, which belong to the TNF receptor superfamily and are characterized by extracellular cysteine-rich domains and extracellular death domains. In other words, the extrinsic pathway is induced by cell death receptor pathways such as TRAIL or FAS ligand. The cell surface receptors form a multiprotein complex called the death-inducing signaling complex (DISC).

The intrinsic pathway, on the other hand, is mediated by mitochondria in response to apoptotic stimuli, such as DNA damage, irradiation and some other anticancer agents (Zhan *et al*., 2012), serum deprivation, cytochrome c, SMAC/DIABLO (a direct inhibitor of apoptosis-binding protein), AIF (apoptosis-inducing factor that promotes chromatin condensation), and EndoG (endonuclease G facilitates chromatin condensation). Cytochrome c binds to and activates Apaf-1 (apoptotic protease activating factor-1) protein in the cytoplasm. This induces the formation of an apoptosome that subsequently recruits the initiator procaspase-9, yielding activated caspase-9, and finally mediates the activation of caspase-3 and caspase-7 (Tan *et al*., 2009). It is apparent that diverse stimuli cause release of mitochondrial proteins to activate the intrinsic apoptosis pathway leading to MOMP and the release of cytochrome c and other apoptogenic proteins; MOMP is regulated by the Bcl family of proteins. In summary, in both pathways activated caspases cleave and activate other downstream cellular substrates as explained above.

Under stress conditions, prosurvival and prodeath processes are simultaneously activated and the final outcome depends on the complex cross-talk between autophagy and apoptosis. Generally, autophagy functions as an early induced cytoprotective response, favoring stress adaptation by removing damaged subcellular constituents. It is also known that apoptotic stimuli induce a rapid decrease in the level of the autophagic factor activating molecule in Beclin 1-regulated autophagy (Ambra 1) (Pagliarini *et al*., 2012). Such Ambra 1 decrease can be prevented by the simultaneous inhibition of caspases and calpains. Caspases cleave Ambra 1 at the D482 site, while calpains are involved in complete Ambra 1 degradation. Ambra 1 levels are critical for the rate of apoptosis induction.

Autophagy can trigger caspase-independent cell death by itself, or by inducing caspase-dependent apoptosis. Autophagy can protect cells by preventing them from undergoing apoptosis. Autophagy also protects cells from various other apoptotic stimuli. Although the exact mechanism underlying this protection is not known, the role of damaged mitochondrial sequestration has been suggested; this prevents released cytochrome c from being able to form a functional apoptosome in the cytoplasm (Thorburn, 2008). There is a close connection between the autophagic machinery and the apoptosis machinery. Is it possible that there is simultaneous activation of these two types of death processes? In fact, autophagy is interconnected with apoptosis, as the two pathways share key molecular regulators (Eisenberg-Lener *et al*., 2009). For example, it has been reported that autophagy regulates neutrophil apoptosis in an inflammatory context-dependent manner, and mediates the early pro-apoptotic effect of TNF-α in neutrophils. Neutrophils are a major subset of circulating leukocytes, and play a central role in defense against bacterial and fungal infections.

The concept of the presence of cross-talk between autophagy and apoptosis is reinforced by the indication that common cellular stresses activate various signaling pathways

which regulate both of these cell death programs. ROS induce apoptosis and regulate Atg4, which is essential for autophagy induction. In addition, Atg5 promotes both apoptosis and autophagy induction. In addition to Atg5, several other signal transduction pathways (Bc12 regulator) can elicit both of those cell death mechanisms. The transcription factor p53 is another such molecule.

Several additional recent studies have revealed additional information regarding the molecular mechanisms underlying the cross-talk between autophagy and apoptosis. An interesting study of the effect of ganoderic acid (a natural triterpenoid) on melanoma cells was recently carried out by Hossain *et al*. (2012). This study indicated that ganoderic acid induced orchestrated autophagic and apoptotic cell death as well as enhanced immunological responses via increased HLA class II presentation in melanoma cells. In other words, this treatment initiated a cross-talk between autophagy and apoptosis as evidenced by increased levels of Beclin 1 and LC3 proteins.

Another study investigated the effect of taurine on methamphetamine (METH)-induced apoptosis and autophagy in PC12 cells, and the underlying mechanism (Li *et al*., 2012). METH, a commonly abused psychostimulant, induces neuronal damage by causing ROS formation, apoptosis, and autophagy. Taurine, in contrast, decreases METH-induced damage by inhibiting autophagy, apoptosis, and oxidative stress through an mTOR-dependent pathway. It is known that mTOR is the major negative regulator of autophagy.

The cross-talk between autophagy and apoptosis is indicated by the involvement of Beclin 1 in both of these programmed cell death types. Autophagy and apoptosis are two dynamic and opposing (in most cases) processes that must be balanced to regulate cell death and survival. Available evidence clearly indicates that cross-talk between autophagy and apoptosis does exist, and that in its presence the former precedes the latter. Also, autophagy may delay the occurrence of apoptosis. Many studies indicate that cancer cells treated with an anticancer drug induce both autophagy and apoptosis. In addition, normal cells exposed to cancer-causing agents tend to invoke defense by inducing both autophagy and apoptosis. Moreover, cancer cells exposed to anticancer agents induce autophagy, but in the absence of autophagy these cells develop apoptosis. This concept is confirmed by a recent study by Li *et al*. (2012), which indicated that oridonin (an anticancer agent) upregulates *p21* (an antitumor gene) expression and induces autophagy and apoptosis in human prostate cancer cells, and that autophagy precedes apoptosis, thus protecting such treated cells from apoptosis by delaying the onset of the latter. To substantiate the above conclusions, several other recently published reports are described below.

Co-regulation of both autophagy and apoptosis using bis-benzimidazole derivatives has been reported (Wang *et al*., 2012). These compounds are potent antitumor agents. The implication is that autophagy and apoptosis act in synergy to exert tumor cell death. In another study, it was shown that low-density lipoprotein receptor-related protein-1 (LRP1) mediates autophagy and apoptosis caused by *Helicobacter pylori* in the gastric epithelial cell line AZ-521 (Yahiro *et al*., 2012). This study also proposes that the cell surface receptor, LRP1, mediates vacuolating cytotoxin-induced autophagy and apoptosis; this toxin induces mitochondrial damage leading to apoptosis. In these cells, the toxin triggers formation of autophagosomes, followed by autolysosome formation. Recently it was reported that death-associated protein kinase (DAPK) induces autophagy in colon cancer cells in response to treatment with histone deacetylase inhibitor (HDACi), while in autophagy-deficient

cells DAPK plays an essential role in committing cells to HDACi-induced apoptosis (Gandesiri *et al*., 2012).

Further evidence supporting the cross-talk between autophagy and apoptosis was recently reported by Visagie and Joubert (2011). They demonstrated the induction of these two programmed cell death mechanisms in the adenocarcinoma cell line MCF-7, which was exposed to 2-methoxyestradiol-bis-sulfamate (2-MeDE2bis MATE), a 2-methoxyestradiol derivative (an anticancer agent). The presence of apoptosis was indicated in this morphological study by growth inhibition, presence of a mitotic block, membrane blebbing, nuclear fragmentation, and chromatin condensation, which are hallmarks of this type of cell death. Simultaneously, this drug induced autophagy, shown by increased lysosomal staining.

Organic compounds have also been used to determine the cross-talk between autophagy and apoptosis. A few examples follow. Pterostilbene (a naturally occurring plant product) activates autophagy and apoptosis in lung cancer cells by inhibiting epidermal growth factor receptor and its downstream pathways (Chen *et al*., 2012). Gui *et al*. (2012) used glyphosate (a herbicide linked to Parkinson's disease) to induce autophagy and apoptosis in PC12 cells, and found that the *Beclin 1* gene was involved in cross-talk between the mechanisms governing the two programmed cell death types. Two plant products, dandelion root extract and quinacrine, mediate autophagy and apoptosis in human pancreatic cancer cells and colon cancer cells, respectively (Ovadje *et al*., 2012; Mohaptra *et al*., 2012). Hirsutanol A compound from the fungus *Chondrostereum* inhibits cell proliferation, elevates ROS level, and induces autophagy and apoptosis in breast cancer MCF-7 cells (Yang *et al*., 2012).

A switch from apoptosis to autophagy is not uncommon during chemoresistance by cancer cells. It is known that defective apoptosis is an important mechanism underlying chemoresistance by cancer cells. Such resistance is associated with profound changes in cell death responses, and a likely switch from apoptosis to autophagy. This switch involves balancing the deletion of multiple apoptotic factors by upregulation of the autophagic pathway and collateral sensitivity to the therapeutic agent. Ajabnoor *et al*. (2012) have reported that reduction of apoptosis occurring in the MCF-7 breast cancer cells upon acquisition of paclitaxel resistance is balanced by upregulation of autophagy as the principal mechanism of cytotoxity and cell death; this sensitivity is associated with mTOR inhibition. Upregulation of the autophagic pathway gives rise to rapamycin resistance. Also, loss of expression of caspase-7 and caspase-9 is observed in these cells.

It is known that the cell survival mechanism is driven by Beclin 1-dependent autophagy, while cell death is controlled by caspase-mediated apoptosis. Both of these processes share regulators such as Bcl2, and influence each other through feedback loops. The question is whether autophagy and apoptosis coexist at the same time at the same stress level. To elucidate the role of regulatory components involved in both autophagy and apoptosis, and better understand the cross-talk between these two programmed cell death mechanisms, Kapuy *et al*. (2013) have explored the systems level properties of a network comprising cross-talk between autophagy and apoptosis, using a mathematical model. They indicate that a combination of Bcl2-dependent regulation and feedback loops between Beclin 1 and caspases strongly enforces a sequential activation of cellular responses depending upon the intensity and duration of stress levels (transient nutrient starvation and growth factor

withdrawal). This study also shows that amplifying loops for caspase activation involving Beclin 1-dependent inhibition of caspases and cleavage of Beclin 1 by caspases not only make the system bistable but also help to switch off autophagy at high stress levels. In other words, autophagy is activated at lower stress levels, whereas caspase activation is restricted to higher levels of stress. Apparently, autophagy precedes apoptosis at lower stress levels, while at a very high stress level apoptosis is activated instantaneously and autophagy is inactivated. According to this observation, autophagy and apoptosis do not coexist at the same time at the same stress level.

In summary, it is clear that a close relationship exists between autophagy and apoptosis, and that autophagy and apoptosis are not mutually exclusive pathways. They can act in synergy, or can counteract or even balance each other. Both share many of the same molecular regulators (Bcl-2). However, stress (e.g., nutrient deficiency, growth factor withdrawal) levels tend to affect autophagy and apoptosis differently from each other, resulting in mutual balancing. Thus, in a clinical setting it is difficult to predict the outcome of inhibition or activation of one form of programmed cell death (autophagy) without considering that of the other (apoptosis) (Eisenberg-Lerner *et al.*, 2009). Because autophagy is involved not only in cell death but also (and mostly) in cell survival, and apoptosis leads only to cell death, an understanding of the critical balance between these two types of cellular processes is required to design anticancer therapeutics. The dual role of autophagy depends on the context and the stimuli. It has even been proposed that not only autophagy and apoptosis but also programmed necrosis may jointly decide the fate of cells of malignant neoplasms (Ouyang *et al.*, 2012).

AUTOPHAGY AND UBIQUITINATION

Ubiquitin is a small (76-amino acid) protein that is highly conserved and widely expressed in all eukaryotic cells. Ubiquitination involves one or more covalent additions to the lysine residues of target proteins. Ubiquitination is a reversible process due to the presence of deubiquitinating enzymes (DUBs) that can cleave ubiquitin from modified proteins. Post-translational modification of cell proteins, including ubiquitin, are involved in the regulation of both membrane trafficking and protein degradation. Ubiquitination is also implicated in the autophagy pathway (Kirkin *et al.*, 2009).

Successful invasion of the host cell by pathogenic microorganisms depends on their ability to subvert intracellular signaling to avoid triggering the cell's immune response. The host cell, under normal conditions, possesses pathways (xenophagy) that protect it from infection. Post-translation modifications (ubiquitination) play a role in the activation of xenophagy. A link between ubiquitination and the regulation of autophagy has been established (Dupont *et al.*, 2010). It is also known that p62 proteins target protein aggregates for degradation via autophagy. Pathogens, however, have developed mechanisms that subvert a cell's defense systems (xenophagy), replicating themselves. *Mycobacterium tuberculosis*, for example, prevents inflammasome activation (Master *et al.*, 2008) Other mechanisms involve interference with the host cell ubiquitination, membrane injury, and impairment of SUMOylation.

AGGRESOME: UBIQUITIN PROTEASOME AND AUTOPHAGY SYSTEMS

The ubiquitin proteasome system (UPS) removes non-functional, damaged, and misfolded proteins from the cell. When the capacity of the proteasome is impaired and/or when the amounts of misfolded proteins exceed the capacity of proteasome, they accumulate in the aggresome, the mechanism of which is explained below. Aggresomes are localized in the proximity of microtubule-organizing center. Microtubule-associated histone deacetylase 6 (HDAC6) mediates this process. Through its ubiquitin-binding BUZ finger domain, HDAC6 binds to and facilitates the transport of polyubiquitinated misfolded proteins along microtubules to aggresome (Kawaguchi *et al.*, 2003). Aggresome removal is mediated by ubiquitin-binding proteins such as p62/SQSTM1 and NBR1. These adaptor proteins through their ubiquitin-binding protein (UBA) are responsible for the fate of protein degradation either through UPS or autophagy (Komatsu and Ichimura, 2010). E3-ubiquitin ligases play a key role in the execution of autophagy (Chin *et al.*, 2010). Recently, it was reported that in response to proteasome inhibition, the E3-ubiquitin ligase TRIM50 localizes and promotes the recruitment and aggregation of polyubiquitinated proteins to the aggresome (Fusco *et al.*, 2012). Fusco and colleagues showed TRIM50 colocalizes, interacts with, and increases the level of p62, which is a multifunctional adaptor protein involved in various cellular processes including the autophagic clearance of polyubiquitinated protein aggregates. The implication of this information is that in the absence of proteasome activity, TRIM50 fails to drive its substrates to proteasome-mediated degradation and promotes their storage in the aggresome for subsequent removal by p62-mediated autophagy. It is known that the accumulation of polyubiquitinated protein aggregates is associated with neurodegenerative disorders and other protein aggregation diseases. It is also known that p62 is a component of inclusion bodies in neurodegenerative diseases and liver diseases.

AUTOPHAGY AND NECROPTOSIS

Necroptosis (type 3 programmed cell death) is one of the three basic cell death pathways. The functions of necroptosis include the regulation of normal embryonic development, T cell proliferation, and chronic intestinal inflammation. The molecular mechanisms underlying TNF-α induced necroptosis and autophagy have been deciphered, and are elaborated below.

Necrostatin-1 (Nec-1), targeting serine–threonine kinase receptor-interacting protein-1 (RIP1), is a specific inhibitor of necroptosis which is dependent on RIP1/3 complex activation (Degtcrev *et al.*, 2008). Tumor necrosis factor alpha (TNF-α) induces necroptosis and autophagy.

It was recently found that TNF-α administration causes mitochondrial dysfunction and ROS production (Ye *et al.*, 2012). Mitochondrial dysfunction led to necroptosis and autophagy in murine fibrosarcoma L929 cells. Nec-1 represses, whereas pan-caspase inhibitor z-VAD-fmk (z-VAD) increases, RIP1 expression. This increase, in turn, enhances TNF-α

induced mitochondrial dysfunction and ROS production. It has also been shown that TNF-α administration and zVAD induce cytochrome c release from mitochondria, whereas Nec-1 blocks this release (Ye *et al.*, 2012).

In addition to apoptosis, necroptosis and autophagy are implicated in controlling both innate and adaptive immune functions. It has been demonstrated that the death of cells following ligation of death receptors (a subfamily of cell surface molecules related to TNF receptor 1) is not exclusively the domain of caspase-dependent apoptosis (Lu and Walsh, 2012). In these cells, cell death occurs via necroptosis.

MITOCHONDRIAL FUSION AND FISSION

Mitochondria form highly dynamic organelles that are continuously fusing and dividing to control their size, number, and morphology. The balance between these two processes regulates their shape. Loss of mitochondrial fusion generates many small mitochondria, while their inability to divide results in elongated mitochondria in most cells (Kageyama *et al.*, 2012).

The central components that mediate mitochondrial dynamics are three conserved dynamin-related GTPases (Kageyama *et al.*, 2011). In mammals, mitochondrial fusion is mediated by mitofusion 1 and 2, and Opal, which are located in the outer and inner membranes, respectively. Mitochondrial division is mediated by Drpl that is mainly located in the cytosol. Drpl is recruited to the mitochondrial surface by other outer membrane proteins (e.g., Mff, MiD49) (Otera *et al.*, 2010; Palmer *et al.*, 2011). The importance of information on functions of Mfn2 and Opal becomes evident considering that mutations in these genes cause neurodegenerative disorders. In other words, alternations in mitochondrial fusion and fission are associated with neurodevelopmental abnormalities.

Mitochondria are highly dynamic cellular organelles involved in a wide variety of physiological functions, including ATP production, apoptosis, calcium and iron homeostasis, aging, lipid metabolism, and the production of reactive oxygen species. Although mitochondria are generally thought to be morphologically static, they alter their morphology continuously in response to various cellular signals, and this phenomenon is termed mitochondrial dynamics (Zungu *et al.*, 2011). These alterations involve mitochondrial division (fission) and the merging of individual mitochondria (fusion). Contact sites between the inner and outer mitochondrial membranes consists of components of the mitochondrial permeability transition pore, which serves as the site for fission and fusion (Reichert and Neupert, 2004).

Under certain starvation conditions (e.g., amino acid depletion) mitochondria may escape autophagosomal degradation through extensive fusion. Such mitochondrial fusion under starvation conditions provides enough ATP necessary for cell survival. Downregulation of the mitochondrial fission protein Drpl is considered to be responsible for the fusion (Rambold *et al.*, 2011a). The process of fusion tends to result in the interconnected mitochondrial network through their elongation. As expected, pharmacological and genetic inhibition of mTOR leads to increased mitochondrial fusion. It is known that mTOR controls mitochondrial fusion. However, other signaling pathways (e.g., AMPK and PKA) may also be involved in starvation-induced mitochondrial fusion (Rambold *et al.*, 2011b).

SELECTIVE AUTOPHAGIES

Specific or selective autophagy requires specific receptors to engage the substrate with the autophagy machinery, such as Atg32 for mitophagy and Atg19 for the cytoplasm to vacuole targeting pathway. Autophagy exhibits significant versatility in its selectivity to degrade cell components, which is discussed below.

Allophagy

In sexual reproduction, gamete fusion leads to the combination of two nuclear genomes, but the fate of paternal mitochondrial DNA requires explanation. Cumulative evidence indicates that in most animals, including humans, paternal mitochondria usually are eliminated during embryogenesis, a process termed allophagy, which is accomplished through autophagy.

A number of mechanisms have been proposed to explain allophagy. Some years ago Gyllenstein *et al.* (1991) hypothesized that according to the "simple dilution model", the paternal mitochondrial DNA (present at a much lower copy number) is simply diluted away by the excess of oocyte mitochondrial DNA, and consequently the former is hardly detectable in the offspring. On the other hand, according to the "active degradative process", the paternal mitochondrial DNA or mitochondria themselves are selectively eliminated (either before or after fertilization) by autophagy, preventing their transmission to the next generation (Al Rawi *et al.*, 2012).

As indicated above, uniparental inheritance of mitochondrial DNA is observed in many sexually reproducing species, and may be accomplished by different strategies in different species. Sato and Sato (2012, 2013) have proposed the following strategies.

1. Diminished content of mitochondrial DNA during spermatogenesis.
2. Elimination of mitochondrial DNA from mature sperms.
3. Prevention of sperm mitochondria from entering the oocyte.
4. Active degradation of the paternal mitochondrial DNA in the zygote.
5. Selective degradation of the whole paternal mitochondria (mitophagy) in the zygote.

The most feasible mechanism to accomplish this goal in mammals is as follows. Sperm-derived mitochondria and their DNA enter the oocyte cytoplasm during fertilization and temporarily coexist in the zygote alongside maternal mitochondria. However, very shortly after fertilization, paternal mitochondria are eliminated from the embryo. Thus, mitochondrial DNA is inherited solely from the oocyte from which mammals develop. This also means that some human mitochondrial diseases are caused by maternal mitochondrial DNA mutations.

The embryo of the *Caenorhabditis elegans* nematode has been extensively used as an experimental model for exploring the role of autophagy in the degradation of paternal organelles (Al Rawi *et al.*, 2012). It has been shown that paternal mitochondrial degradation depends on the formation of autophagosomes a few minutes after fertilization. This macroautophagic process is preceded by an active ubiquitination of some spermatozoon-inherited organelles, including mitochondria. The signal for such degradation is polyubiquitination of paternal mitochondria. Sato and Sato (2012) have also reported selective allophagy in such embryos.

It should be noted that the elimination of paternal mitochondrial DNA is not universal. Paternal inheritance of mitochondrial DNA, for example, has been reported in sheep and lower primates (St. John and Schatten, 2004; Zhao *et al.*, 2004). A recent study using mice carrying human mitochondrial DNA indicated that this DNA was transmitted by males to the progeny in four successive generations, confirming the paternal transmission of mitochondrial DNA (Kidgotko *et al.*, 2013). Apparently, human mitochondrial DNA safely passed via the male reproductive tract of several mice in several generations. This and a few other studies invoke a question regarding the existence of a specific mechanism responsible for paternal mitochondrial DNA transmission. Another pertinent, more important, unanswered question is: why are paternal mitochondria and/or their DNA eliminated from embryos? One hypothesis is that paternal mitochondria are heavily damaged by ROS prior to fertilization, and need to be removed to prevent potentially deleterious effects in the next generation (Sato and Sato, 2013).

Glycophagy

The delivery of glycogen to lysosomes for degradation is termed glycophagy. Three types of enzymes convert glucose into uridin diphosphoglucose, the primary intermediate in glycogen synthesis. The glucose residue of the intermediate molecule is transferred by glycogen to the free hydroxyl group on carbon 4 of a glucose residue at the end of a growing glycogen chain. Glycogen functions as a reserve for glucose, and provides an intracellular energy reserve in many types of cells. Glycogen is especially abundant in liver and muscle cells. As much as 10% by weight of the liver can be glycogen. The presence of glycogen particles in the vicinity of the smooth endoplasmic reticulum membranes in the liver as well as in the sarcoplasmic reticulum membranes in muscle is commonly seen using electron microscopy (Hayat, personal observation). Glycogen is also present in lysosomes of mammalian cells where it is directly hydrolyzed by lysosomal acid alpha-glucosidase (acid maltase). Deficient glucosidase causes severe glycogen storage diseases (Pompe disease, cardiopathologies).

Normally, synthesis and degradation of glycogen are highly regulated according to need. Accumulation of glycogen tends to cause a severe glycogen storage disease, Pompe disease, in multiple tissue types, especially in skeletal and cardiac muscles. The build up of glycogen forms a large mass that interrupts the contractile proteins of the skeletal muscle fibers, affecting muscle contraction (Fukuda *et al.*, 2006), muscular weakness, and eventual tissue destruction. Other glycogen diseases include Anderson disease (Chen and Burchell, 1995), Tarui disease (Nakajima *et al.*, 1995), and Lafora disease (Andrade *et al.*, 2007).

Some information is available explaining glycogen trafficking to the lysosomes and its degradation. Autophagy seems to be involved in this process. The starch-binding domain-containing protein 1 (Stbd 1) (genethonin 1) participates in this mechanism by anchoring glycogen to intracellular membranes via its N-terminus (Janecek, 2002; Jiang *et al.*, 2011). Degradation of glycogen occurs by removing glucose residues catalyzed by glycogen phosphorylase. Stbd 1 targets two autophagy-related proteins, GABARAP and GABARAPL 1. Stbd 1 acts as a cargo receptor for glycogen. The Atg8 family interacting motif (AIM) in Stbd 1 is responsible for its interaction with GABARAPL 1 (Jiang *et al.*, 2011). Stbd 1 is thought to function as a cargo binding protein that delivers glycogen to lysosomes in an autophagic pathway (glycophagy). In fact, Stbd 1 is considered to be a glycophagy marker.

Lipophagy

The vast majority of studies of autophagy in the past rightfully have emphasized its role in cellular energy balance, cellular nutritional status, cellular quality control, remodeling, and cell defense. In most of these studies emphasis was placed on the role of autophagy in supplying energy through degradation of proteins to obtain amino acids required to maintain protein synthesis under extreme nutritional conditions. However, the contribution of autophagy to maintain cellular energetic balance is not solely dependent on its capacity to provide free amino acids (Singh and Cuervo, 2012). Free amino acids are relatively inefficient source of energy when oxidized to urea and carbon dioxide. In contrast, free fatty acids and sugars are more efficient in supplying energy, especially the former through lipophagy.

Lipophagy is a selective form of autophagy and refers to the degradation of lipid droplets by stimulating autophagy. Lipid droplets are intracellular storage deposits for neutral lipids that are widely present in cells ranging from bacteria to humans. These droplets are considered to be organelles enclosed by a polar lipid monolayer membrane. They contain the hydrophobic core of triglycerides, diacyglycerol, cholesterol ester, and other esters. Mobilization of lipids inside the lipid droplets occurs through lipolysis. Cells activate lipolysis when they need energy and also when lipid storage becomes too large. The synthesis of fatty acids and phospholipids occurs in the smooth endoplasmic reticulum (SER).

Autophagy has been implicated in the degradation of several types of intracellular components, but only relatively recently have cytoplasmic lipid droplets been added to the list. This process of lipophagy has raised the likelihood that autophagy is involved in the regulation of lipoprotein assembly and contributes to both intracellular and whole-body lipid homeostasis (Christian *et al.*, 2013). Thus, autophagy is thought to be partially responsible for the upregulation or downregulation of very low density lipoprotein (VLDL) assembly. This means that autophagy is involved in the regulation of lipid accumulation during adipocyte differentiation.

Lipophagy breaks down triglycerides and cholesterol stored in lipid droplets, regulating intracellular lipid content. This degradation supplies free fatty acids required to sustain cellular rates of mitochondrial levels of ATP. In other words, lipophagy maintains cellular energy homeostasis. Intracellular lipids, in addition, function as structural components of membrane building blocks for hormones, and mediators of cell signaling. The amount of lipid targeted for autophagic degradation depends on the nutritional status.

Another important function of autophagy is in liver diseases which are characterized by the accumulation of triglycerides and irregular lipid metabolism within the liver. It has been reported that suppression of autophagy pathway leads to the accumulation of lipid droplets in hepatocytes and other cell types (Singh *et al.*, 2009).

Aberrant autophagy is also involved in conditions of deregulated lipid homeostasis in metabolic disorders such as metabolic syndrome of aging (Christian *et al.*, 2013). Lipophagy is also functionally involved in hypothalamic neurons and macrophage foam cells (Kaushik *et al.*, 2011; Ouimet and Marcel, 2012). A variety of proteins (Rab and PAT) are also associated with the lipid droplet membrane. PAT proteins regulate cytosolic lipase-mediated lipolysis, a major pathway for regulating lipid homeostasis (Fujimoto *et al.*, 2008). Impaired lipophagy, indeed, is a fundamental mechanism of disorders of lipid metabolism such as obesity, diabetes, and atherosclerosis. The initial accumulation of excess lipid is referred to as steatosis (Czaja, 2010).

The role of lipophagy in the alcohol-induced liver is discussed later. In addition to the role played by lipophagy in the above mentioned diseases, the role of lipid accumulation in cardiovascular diseases was recently studied by Kim *et al.* (2013). Epigallocatechin gallate (EGCG) is a major polyphenol in green tea, which has beneficial health effects in the prevention of cardiovascular disease. These authors suggest that EGCG regulates ectopic lipid accumulation through a facilitated lipophagy flux. Treatment with EGCG increases the formation of LC3-II and autophagosomes in bovine aortic endothelial cells. Activation of CaMKKβ is required for EGCG-induced LC3-II formation. This effect is due to cytosolic C++ load. It is concluded that EGCG induces lipophagy through a reduction in the accumulation of lipid droplets in endothelial cells. It is known that impairment of the lysosomal degradation process reduces autophagic flux leading to serious disorders in cardiovascular and metabolic tissues (Singh and Cuervo, 2011).

The following questions still remain to be answered and open for future studies (Singh and Cuervo, 2012):

1. Is there any similarity between the signaling pathways that regulate lipophagy and those for other types of autophagy?
2. What is the molecular mechanism underlying the selective targeting of the lipid droplets by lipophagy?
3. Is there a subset of lipid droplets that is targeted by lipophagy?
4. Is there a difference between the lipid products by produced by lipophagy and those arising from lipolysis?
5. How does the switch take place from a stimulatory to an inhibitory effect of free fatty acids on lipophagy?
6. Does upregulation of lipophagy protect cells from lipotoxicity?
7. Does defective hypothalamic lipophagy contribute to the reduced food intake at an advanced age?
8. What is the potential of developing a therapeutic intervention against metabolic disorders by organ-specific targeting of this process?

Role of Lipophagy in Alcohol-Induced Liver Disease

An interesting role of lipophagy and mitophagy in chronic ethanol-induced hepatic steatosis has been reported (Eid *et al.*, 2013). It is known that chronic alcohol intake may induce alcoholic disease, ranging from early-stage steatosis (fatty liver) to steatohepatitis, fibrosis, cirrhosis, and finally hepatic cancer (Yan *et al.*, 2007). Rats fed with 5% ethanol in liquid diet for 10 weeks showed large lipid droplets and damaged mitochondria in steatolic hepatocytes (Eid *et al.*, 2013). Moreover, hepatocyte steatosis was associated with enhanced autophagic vacuole formation compared to control hepatocytes. In addition, LC3 (a marker for autophagosomes) demonstrated an extensive punctate pattern in hepatocytes of these experimental rats.

Furthermore, PINK1 (a sensor damaged mitochondria, mitophagy) as well as LAMP-2 (a marker of autolysosomes) were expressed in these rats. This information provides clear evidence of ethanol toxicity because of the accumulation of lipid droplets in the cytoplasm of hepatocytes involving lipogenesis and lipolysis. Elevated levels of lipophagy and mitophagy reduce hepatocyte cell death under acute ethanol toxicity (Ding *et al.*, 2011).

In conclusion, the enhanced autophagic sequestration of accumulated lipid droptes presence of endogenous LC3-II, LAMP-2, PINK 1, pan cathepsin, and cytochrome c under chronic ethanol toxicity. Nevertheless, the available information is insufficient to explain the relationship between lipophagy and canonical autophagy as well as between lipophagy and cytosolic lipolysis. The deciphering of the molecular mechanism underlying such differences may provide new therapeutic tools.

Mitophagy

It is thought that after its endosymbiosis from an α-proteobacterial ancestor, the mitochondrial genome was streamlined into a small, bioenergetically specialized genetic system, allowing an individual mitochondrion to respond through gene expression to alterations in membrane potential and maintain oxidative phosphorylation. Replication and transcription of mitochondrial DNA is initiated from a small noncoding region, and is regulated by nuclear-encoded proteins that are post-translationally imported into mitochondria. Mitochondria possess a unique genetic system that is able to translate the mitochondria-encoded genes into 13 protein subunits of the electron chain. Mercer *et al.* (2011) have presented analysis of the mitochondrial transcription across multiple cell lines and tissues, revealing the regulation, expression, and processing of mitochondrial RNA. This information should help in the understanding of exceedingly complex function of mitochondria. The major functions of mitochondria are summarized below.

Mitochondria fulfill central roles in oxidative phosphorylation, and energy metabolism, in the synthesis of amino acids, lipids, heme, and iron sulfur clusters, in ion homeostasis and in thermogenesis. The most important role of mitochondria is to provide energy to aerobic eukaryotic cells by oxidative phosphorylation. Thus, these organelles are essential for growth, division, and energy metabolism in these cells. Each cell usually contains hundreds of mitochondria, and without these organelles even cancer cells are unable to grow, multiply, and survive *in vivo*. Mitochondrial dysfunction is strongly linked to numerous neurodegenerative and muscular disorders, myopathies, obesity, diabetes, cancer, and aging. Minimizing mitochondrial dysfunction is thus of major importance for counteracting the development of numerous human disorders and the aging process.

Mitochondria also play a crucial role in apoptosis and autophagy. It is apparent that mitochondria are central to the two fundamental processes of cell survival and cell death. Mitophagy plays a major role in the specific recognition and removal of damaged mitochondria, and thus in mitochondrial quality control. The quality control of mitochondria does occur naturally at different levels. On the molecular level dysfunctional mitochondria are recognized and degraded within cells by autophagy. Mitochondria can be degraded both by nonselective autophagy and by mitophagy. Engulfment of mitochondria by autophagosomes is observed under starvation conditions as well as when mitochondrial function is impaired.

Mitochondrial turnover is necessary for cellular homeostasis and differentiation. Mitochondria are replaced every 2–4 weeks in rat brain, heart, liver, and kidney. The removal of dysfunctional mitochondria is achieved through mitophagy. Mitophagy is responsible for the removal of mitochondria during terminal differentiation of red blood cells and T cells. Mitochondria are recognized for selective mitophagy either by PINK1 and Parkin or mitophagic receptors Nix and Bnip3 and their accompanying modulators

(Novak, 2012). The former mitophagy recognizes mitochondrial cargo through polyubiquitination of mitochondrial proteins. Nix functions as a regulated mitophagy receptor. These two modes of capturing mitochondria function at different efficiencies, from partial to complete elimination of mitochondria. In addition to autophagy machinery, proteins associated with mitochondrial fusion and fission regulate mitochondrial morphology, which is discussed elsewhere in this chapter.

A number of factors required for mitophagy have been identified and their role in this process has been analyzed. NIX (a BH3 domain containing protein) acts as a mitochondrial receptor required for mitochondrial clearance in some types of cells (e.g., reticulocytes). Many studies have shown that PINK1 and Parkin are involved in mitophagy. Mitochondrial depolarization induced by protonophore CCCP, downregulation of PINK1, and ROS induce mitophagy as well as non-selective autophagy. More importantly, mitochondrial fission is necessary for the induction of mitophagy.

Nucleophagy

Parts of the cell nucleus can be selectively degraded without killing the cell, by a process termed nucleophagy. The cell nucleus is an organelle bounded by a double membrane, which undergoes drastic reorganization during major cellular events such as cell division and apoptosis. The process of nucleophagy is best described in the budding yeast, *Saccharomyces cerevisiae*. Under certain conditions, the removal of damaged or non-essential parts of the nucleus or even an entire nucleus (differentiation or maturation of certain cells) is necessary to promote cell longevity and normal function; such degradation and recycling are accomplished via nucleophagy (Mijaljica and Devenish, 2013). Autophagic degradation of the nucleus in mammalian cells as a "housecleaning" under normal and disease conditions has been studied (Mijaljica *et al.*, 2010).

Molecular mechanisms underlying the formation of nucleus–vacuole junctions that mediate nucleophagy in the yeast have been deciphered. This mediation is accomplished through specific interactions between Vac8p on the vacuole membrane and Nvj1p in the nuclear envelope. Electron microscopy has shown that portions of the nucleolus are sequestered during nucleophagy (Mijaljica *et al.*, 2012).

Morphologically, during nucleophagy, a nuclear bleb containing the nuclear cargo is pinched off from the nucleus and directly engulfed and sequestered into an invagination of the vacuolar membrane rather than packaged into autophagosome-like vesicles. It has been shown that upon nitrogen starvation the initiation of piecemeal micronucleophagy of the nucleus (PMN) occurs, as stated above, at the nucleus–vacuole junction between the outer nuclear membrane protein, Nvj1p, and the vacuolar membrane protein, Vac8p. Recently, it was demonstrated that induction of PMN can be detected as early as after 3 h of nitrogen starvation (Mijaljica *et al.*, 2012). Mijaljica and co-workers employed genetically encoded nuclear fluorescent reporters (n-Rosella).

The PMN occurs through a series of morphologically distinct steps: (1) a nucleus–vacuole junction is formed at the nuclear envelope (both inner and outer membranes are involved); (2) simultaneous invagination of the vacuolar lumen occurs; (3) the nuclear derived double membranous structure containing nuclear material undergoes fission and is degraded by vacuolar hydrolases. This efficient process requires core ATG genes. All four components of

the Atg8p–phosphatidylethanolamine conjugation system (ATG3, ATG4, Atg7, and ATG8) have been reported to be essential for efficient late nucleophagy.

The role of lipid trafficking membrane proteins in the mechanism of late nucleophagy is important. Kvam and Goldfarb (2004) have proposed that yeast Osh proteins play a general role in lipid trafficking at membrane contact sites between different organelles including the nucleus and vacuole. Roberts *et al.* (2003) have shown that upon nitrogen starvation and concomitant increased expression of Nvj1p, two proteins – Osh1- and Tsc13p – were required for PMN. In spite of the known molecular mechanisms discussed above, the specific conditions under which various cell nucleus components such as nucleoli, chromosomes, chromatin, histones, nuclear pore complexes, and nucleoplasm are degraded are not known.

Pexophagy

The selective degradation of peroxisomes by autophagy is referred to as pexophagy. The number of peroxisomes in a cell is tightly regulated in response to changes in metabolic status. They can be rapidly and selectively degraded when methanol-grown cells are placed in conditions of repression of methanol metabolism (e.g., glucose) by a process termed micropexophagy (van Zutphen *et al*., 2008). Degradation of peroxisomes is also observed when the cells are placed in an ethanol medium; this is termed macropexophagy. In other words, micropexophagy is induced by glucose, and macropexophagy is induced by ethanol. The micro- and macropexophagy pathways are morphologically similar to the micro- and macroautophagy pathways, respectively. On the other hand, phthalate esters can cause a marked proliferation of peroxisomes. It has been demonstrated in yeast that protein trafficking, lipid trafficking, or both as directed by Sar1p are essential for micro- and macropexophagy (Schroder *et al*., 2008). Stasyk *et al*. (2008) have presented methods for monitoring peroxisome status in yeast. Autophagic degradation of peroxisomes can be monitored with electron microscopy as well as by using biochemical assays for peroxisome markers. Several types of membrane dynamics during pexophagy can be visualized simultaneously under live cell imaging.

Pexophagy has been extensively studied in the methylotrophic yeast *Pichia pastoris*, which is capable of growth on methanol as a sole source of carbon and energy. There are two types of pexophagy: (1) micropexophagy through microautophagy; and (2) macropexophagy through macroautophagy. The induction of these two pathways depends on the carbon source in the methylotrophic yeast (Ano *et al*., 2005). Micropexophagy is induced by glucose, and macropexophagy by ethanol. During micropexophagy, peroxisomes are incorporated directly into the vacuoles by invagination; during macropexophagy, in contrast, peroxisomes are sequestered primarily by inclusion within newly-formed membranes. Subsequently, the peroxisome-containing pexophagosome fuses with the vacuole to deliver its cargo. Micropexophagy is more sensitive to ATP depletion than is macropexophagy, implying that former process requires a higher level of ATP.

It has been shown in yeast that PpAtg9 is essential for formation of the sequestering membranes that engulf the peroxisomes for degradation within the vacuole (Chang *et al*., 2005). Upon the onset of micropexophagy, PpAtg11 recruits PpAtg9 to the perivascular structure, which acts as the site of formation of the sequestering membrane presumably by causing segmentation of the vacuole. These membranes subsequently engulf the peroxisomes and eventually fuse with the help of PpAtg1 and PpVac8 to incorporate the peroxisomes into the

vacuole for degradation (Chang *et al.*, 2005). In the light of the difference in the sequestering mechanism between micropexophagy and macropexophagy, the former process requires a higher level of ATP.

Reticulophagy

Reticulophagy is responsible for the selective sequestration of portions of the endoplasmic reticulum (ER) with associated ribosomes. ER is a highly complex organelle, composed of a single continuous phospholipid membrane and flattened peripheral sheets with associated ribosomes. Almost all eukaryotic cells contain a discernible amount of ER because it is needed for the synthesis of plasma membrane proteins and proteins of the extracellular matrix. While detoxification of drugs, fatty acid and steroid biosynthesis, and Ca^{2+} storage occur in the smooth ER, most of the folding and post-translational processing of membrane-bound and secreted proteins take place in the ER. Ribosomes that are present free in the cytosol mainly translate cytoplasmic proteins, whereas ribosomes associated with the ER membrane synthesize proteins that are secreted or reside in one of the organelles of the endomembrane system. As these newly synthesized proteins are cotranslationally translated into the ER, a substantial proportion of these proteins remain located in this compartment (Cebollero *et al.*, 2012).

The ER stress signal, along with other signals (e.g., oxidative signal), is involved in autophagy. The former is involved in membrane formation and fusion, including autophagosome formation, autophagosome-lysosome, and degradation of intra-autophagosomal contents by lysosomal hydrolases. ER stress is also involved in amplifying ROS production (Rubio *et al.*, 2012). The study by Rubio *et al.* (2012) indicated that apical ER photodamage in murine fibrosarcoma cells generated ROS via mitochondria, which contributed to the processes of reticulophagy.

Unfolded protein response (UPR) is an intracellular signaling triggered by the ER stress. ER stress occurs under various physiological and pathological conditions where the capacity of the ER to fold proteins becomes saturated, for example as a response to incompetent or aggregation prone proteins, Ca^{2+} flux across the ER membrane, glucose starvation, or defective protein secretion or degradation (Hoyer-Hansen and Jaattela, 2007). Glucose starvation results in reduced protein glycosylation, and hypoxia causes reduced formation of disulfide bonds. ER stress resulting from the accumulation of unfolded or misfolded proteins threatens cell survival and the ER to nucleus signaling pathway; this pathway is called the UPR. UPR reduces global protein synthesis and induces the synthesis of chaperone proteins and other proteins, which increase the ER capacity to fold its client proteins (Hoyer-Habsen and Jaattela, 2007). To prevent the accumulation of misfolded polypeptides in the ER, chaperone proteins are thought to assist in the folding of the nascent polypeptides or recognize the misfolded proteins and mediate their refolding (Braakman and Bulleid, 2011). However, under certain conditions, unfolded proteins accumulate in the ER. At least two interconnected mechanisms are available to cope with such undesirable protein aggregation: (1) the UPR and (2) the ER-associated degradation (ERAD) (Bernales *et al.*, 2006; Romisch, 2005).

The UPR signaling is transduced into cytoplasmic and nuclear actions aimed at increasing the protein folding capacity of the ER and eliminating the proteins that remain misfolded and accumulated in the ER. UPR also initiates inhibition of general translation

and upregulation of genes encoding ER chaperones and components of the ERAD machinery (Cebollero *et al.*, 2012). The ERAD, in turn, recognizes misfolded proteins and translocates them into the cytoplasm where they are degraded by the ubiquitin-proteasome system. When the function of the ER is not restored, it may lead to cell death by apoptosis or autophagy depending on the cell type and the stimulus (Momoi, 2006).

Ribophagy

Selective degradation of ribosomes is termed ribophagy. Ribosomes are essential components of all cells and constitute the translation engine of the cell. Protein synthesis is catalyzed by ribosomes, which are composed of large complexes of RNA and protein molecules. Each ribosome is composed of one large subunit (60S) and one small subunit (40S) in eukaryotes, while prokaryotic ribosomes are made up of 50S and 30S subunits. Although these two types of ribosomes differ in size and number in eukaryotes and prokaryotes, both have the same function. Before protein synthesis can begin, the corresponding mRNA molecule must be produced by DNA transcription. This is followed by the binding of the small subunit to the mRNA molecule at a start codon that is recognized by an initiator tRNA molecule. Then the large subunit binds to complete the ribosome, and initiates the elongation phase of protein synthesis.

Ribosome turnover occurs both under normal conditions and under starvation. Under normal nutrient-rich conditions, large amounts of ribosomal subunits are assembled, which raises the possibility for the need of the removal of excess ribosomes in response to changing environmental conditions (Bakowska-Zywicka *et al.*, 2006). The ribophagy pathway could also target defective ribosomes under normal growth conditions (Cebollero *et al.*, 2012). This is a quality control function. It is also known that the autophagy of ribosomal proteins is involved in antibacterial function. Some information on the pathway of normal ribosome turnover, especially the role of rRNA decay, is available. *Arabidopsis* RNS2 (a conserved ribonuclease of the RNAse T2 family) is necessary for normal decay of rRNA. The absence of RNS2 results in longer-lived rRNA and its accumulation in the yeast vacuoles and ER, showing constitutive autophagy. This evidence supports the concept that RNS2 participates in a ribophagy-like mechanism that targets ribosomes for recycling under normal growth conditions.

Regarding the role of ribophagy during starvation, cells are subjected to energy shortage and need to save available energy. The beginning of the construction of ribosomes in the cell nucleus and the subsequent translation they carry out require considerable energy. Therefore, cells need to save energy, which is accomplished by removing ribosomes and terminating translation and protein synthesis. Ribophagy begins by separating the two subunits of a ribosome. It has also been suggested that Ubp3/Bre5 (discussed later) regulates different types of selective autophagies during starvation.

It is important to identify the genes required for ribophagy. Kraft *et al.* (2008) indicated the involvement of two proteins, ubiquitin-specific protease 3 (Ubp3) enzyme and Ubp3-associated cofactor (Bres) in the selective degradation of ribosomes, but not for bulk autophagy. They also indicated that ribophagy affects the entire 60S subunit, but not the 40S subunit, suggesting differential degradation of large and small subunits. These authors, furthermore, demonstrated the involvement of Atg1 and Atg7 in the transport of ribosomes to the vacuole in the yeast *S. cerevisiae*. It also has been reported that the Ubp3/Bre5 complex interacts with Atg19 protein and modulates its ubiquitination.

It is concluded that ribosome degradation relies on both ribophagy and non-selective autophagy. The evidence presented there and from other studies confirms a cross-talk between selective autophagy and ubiquitin-dependent processes. The majority of cellular proteins and most other cell components are eventually degraded and recycled in a cell either by autophagy or the ubiquitin–proteasome pathway or by a combination of these two systems. In fact, there is a connection between autophagy and ubiquitin modification and destruction by the proteasome pathways of protein degradation.

Xenophagy

The successful invasion of the host cell by pathogenic microorganisms depends on their ability to subvert intracellular signaling to avoid triggering the cell's immune response. The host cell, under normal conditions, possesses pathways (xenophagy) that protect it from infection. Post-translation modifications (ubiquitination) play a role in the activation of xenophagy. A link between ubiquitination and the regulation of autophagy has been established (Dupont *et al.*, 2010). It is also known that p62 proteins target protein aggregates for degradation via autophagy. Pathogens, however, have developed mechanisms that subvert the cell's defense systems (xenophagy), replicating themselves. *Mycobacterium tuberculosis*, for example, prevents inflammasome activation (Master *et al.*, 2008). Other mechanisms involve the interference with the host cell ubiquitination, membrane injury, and impairment of SUMOylation.

Zymophagy

Pancreatic acinar cells are highly differentiated cells which synthesize and secrete digestive enzymes into the pancreatic juice. These digestive enzymes are initially produced as inactive enzymes (zymogens) and stored in zymogen granules until exocytosis. These granules can be harmful if activated prematurely because the release of these enzymes can hydrolyze tissue parenchyma, resulting in pancreatitis (Grasso *et al.*, 2011). VMP1 interacts with Beclin 1/Atg6 through its hydrophilic C-terminal region, which is necessary for early steps of autophagosome formation. Thus, the involvement of VMP1 is implicated in the induction of autophagy during this disease. VMP1 also interacts with the ubiquitin specific proteases (USPs), indicating close cooperation between the autophagy pathway and the ubiquitin machinery required for selective autophagosome formation (Grasso *et al.*, 2011). Ubiquitination and ubiquitin-receptors such as p62 (SQSTAM1) play a part in vesicular traffic in pancreatits. In fact, a VMP1-USP4-p62 molecular pathway is involved in mitophagy.

As explained above, if zymogen granules prematurely release the digestive enzymes in the acinar cells, the result could be pancreatitis. Under normal physiological conditions selective autophagy (zymophagy) degrades the activated zymogen granules, avoiding the release of digestive enzymes into the cytoplasm and thus preventing further trypsinogen activation and cell death. In other words, zymophagy has a critical function in secretory homeostasis and cell response to injury by selective degradation of altered secretory granules in acute pancreatitis.

In conclusion, zymophagy protects the pancreas from self-digestion. It is a selective form of autophagy, a cellular process to specifically detect and degrade secretory granules

containing activated enzymes before they can digest the organ (Vaccaro, 2012). Zymophagy is activated in pancreatic acinar cells during pancreatitis-induced vesicular transport alteration to sequester and degrade potentially deleterious, activated zymogen granules.

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CHAPTER

2

Molecular Process and Physiological Significance of Mitophagy

Ke Wang and Daniel J. Klionsky

OUTLINE

Abstract

Mitochondria are essential organelles, and their dysfunction could cause severe consequences including aging and the pathogenesis of neurodegenerative diseases. Maintaining a healthy and functional population of mitochondria is critically important for all eukaryotic cells. Despite the fact that several quality control systems within mitochondria have been well characterized from previous research, recent studies have established an important link between mitochondrial maintenance and macroautophagy, which is a highly conserved catabolic process responsible for the degradation of cellular components. Accumulated evidence has demonstrated that autophagy plays an important role in the selective elimination of superfluous and dysfunctional mitochondria. This process is specifically termed mitophagy. At the molecular level, a group of proteins has been identified in various model organisms to mediate the association of the degrading mitochondria with the autophagic machinery. Diverse mechanisms have been reported to ensure

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the efficient and selective recognition and degradation of unwanted mitochondria through macroautophagy, while simultaneously maintaining tight regulation of these degradative processes. The recent advances in our understanding of mitophagy will provide essential insights into the pathogenesis of a variety of mitochondrial dysfunction-related diseases.

INTRODUCTION

Macroautophagy (hereafter referred to as autophagy) is a well-conserved cellular degradation mechanism among eukaryotic organisms from yeast to mammals (Yang and Klionsky, 2010). Extensive studies on autophagy in the past decade greatly expanded our understanding of this cellular pathway from both the perspective of molecular mechanism and biological significance. The hallmark of the process of autophagy is the formation of the double-membrane vesicles called autophagosomes (Xie and Klionsky, 2007). Upon autophagy induction, an initial membrane structure termed the phagophore, the precursor to the autophagosome, gradually expands and engulfs a portion of the cytoplasm or specific cargos and delivers them to the vacuole/lysosome for degradation (Xie and Klionsky, 2007). Autophagy occurs constitutively and operates as a homeostatic mechanism. In addition, autophagy can also be activated in response to a variety of physiological and pathological stress stimuli. The mechanism of autophagosome formation, which includes the sequential expansion of the phagophore, provides autophagy with the capacity to sequester essentially any cellular component and deliver it into the vacuole/lysosome for degradation. Indeed, autophagy has been documented to play a role in degrading a wide range of cellular components, such as protein complexes, the endoplasmic reticulum, peroxisomes, ribosomes, and mitochondria.

Our understanding of autophagy at the molecular level has largely benefited from genetic screens for defects in this pathway in the yeast *Saccharomyces cerevisiae*. To date, nearly 40 autophagy-related (*ATG*) genes have been found to play important roles in this process. At least half of these genes are highly conserved in mammalian systems. Mechanistically, the process of autophagy begins with the activation of the Atg1 protein kinase in yeast, or the ULK1 protein complex in mammals, which integrates the initial inducing signal to counter the negative inhibition by regulators such as TOR complex 1, which otherwise maintain autophagy at the basal level. The formation of the autophagosome proceeds through the subsequent activation of a class III phosphatidylinositol 3-kinase (PtdIns3K) complex that includes Vps30/Atg6 in yeast or BECN1 in mammals. PtdIns3K-mediated formation of the phosphatidylinositol 3-phosphate (PtdIns3P)-rich membranes is important for the nucleation of the phagophore. Atg9-containing vesicles shuttle between the phagophore assembly site (PAS) and putative membrane sources, including the endoplasmic reticulum, Golgi apparatus, plasma membrane or mitochondria for the delivery of membranes and expansion of the phagophore. Atg8–PE conjugates (or LC3–PE [LC3-II] conjugates in mammals) and the Atg12–Atg5-Atg16 complex (or ATG12–ATG5-ATG16L1 in mammals) contribute to the elongation of the phagophore. Atg8–PE or LC3-II is incorporated into both sides of the phagophore, and is required for the elongation of the membrane for the formation of a mature autophagosome (reviewed in Inoue and Klionsky, 2010; Rubinsztein *et al.*, 2012). These protein complexes function in a specific sequence and form part of the core machinery of autophagy that operates in every different type of general and selective autophagic process.

SELECTIVE AUTOPHAGY IN YEAST

Although autophagy is generally considered to be a nonselective bulk degradation pathway, research in this field continues to uncover examples of the importance of selective modes of sequestration. The first identified selective autophagy cargo was the vacuolar aminopeptidase I (Ape1). Ape1 is synthesized in a precursor form (prApe1) and subsequently oligomerizes to form a dodecamer, which continues to assemble into a larger Ape1 complex (Kim *et al.*, 1997). The formation of the mature, and thus functional form, of Ape1 requires the delivery of the Ape1 complex (which is now termed the cytoplasm-to-vacuole targeting [Cvt] complex after binding of the receptor Atg19) to, and processing of prApe1 within, the vacuole. The biosynthetic Cvt pathway occurs constitutively under nutrient-rich conditions. A genetic screen to identify mutants defective in the maturation of prApe1 revealed that the Cvt pathway shares most of the same machinery with the autophagy pathway (Harding *et al.*, 1995). In addition, electron microscopy studies revealed that the Cvt complex is enclosed in double-membrane vesicles that resemble autophagosomes; however, these Cvt vesicles are smaller and exclude most other cytoplasmic components (Baba *et al.*, 1997), strongly suggesting that the Cvt pathway is a type of autophagy that enables the selective sequestration of cargo.

Among the mutants discovered in the Cvt pathway screen was *atg11*, which encodes a scaffold protein (Yorimitsu and Klionsky, 2005). Subsequent studies demonstrated that Atg11 is a common scaffolding protein for a variety of selective autophagic pathways in yeast. In contrast, Atg19 is specific to the Cvt pathway (Shintani *et al.*, 2002). Atg19 binds prApe1, and this interaction is required for association with Atg11, via the latter's binding to the receptor. This association between Atg19 and Atg11 therefore mediates the recognition of the Cvt complex by the core autophagic machinery; Atg19 subsequently binds Atg8–PE, initiating the sequestration of the Cvt complex (Shintani *et al.*, 2002). Neither Atg11 nor Atg19 are required for the progression of nonselective autophagy (and therefore are not considered to be part of the "core" autophagy machinery); thus, these proteins are considered as specific adaptations of the core machinery to allow the sequestration of particular cargos, in this case the Cvt complex.

In addition to the Cvt pathway, Atg11 also mediates other types of selective autophagy through its interaction with different receptor proteins. For example, peroxisome degradation by selective autophagy, termed pexophagy, requires the receptor protein Atg36 in *Saccharomyces cerevisiae* (Motley *et al.*, 2012) or PpAtg30 in the case of *Pichia pastoris* (Farre *et al.*, 2008). The mitochondrion is another organelle that can be the cargo of selective autophagy, and Atg32, a mitochondrial outer membrane protein, is the receptor that is required for this process; similarly to Atg19 (and Atg36 or PpAtg30), Atg32 binds both Atg11 and Atg8–PE (Kanki *et al.*, 2009; Okamoto *et al.*, 2009). In general, this type of ligand-receptor-scaffold system represents a common model for the mechanism of selective autophagy in yeast cells (although in the case of yeast mitophagy a separate ligand is not required since Atg32 is a resident mitochondrial protein).

As indicated previously, the various receptor proteins also interact with Atg8–PE. These receptor proteins contain a well-conserved WXXL sequence that functions as the Atg8-family interacting motif, which is equivalent to the LC3-interacting region (LIR) in mammalian cells. The interactions of the receptor proteins with Atg8 may play auxiliary roles (Kondo-Okamoto *et al.*, 2012), but are not essential for the progression of selective autophagy.

Not all types of selective autophagy are mediated through Atg11. Ald6, a soluble cytoplasmic enzyme, is preferentially degraded by autophagy after nitrogen starvation for an extended time (Onodera and Ohsumi, 2004). The degradation of Ald6 requires the core autophagic machinery, but not Atg11; however, the mechanism used to selectively target Ald6 is not known.

Ribophagy, the selective degradation of ribosomes upon prolonged nutrient deprivation, is another type of selective autophagy. Ribophagy involves the ubiquitination of the ribosomal subunit Rpl25 (Kraft *et al.*, 2008). In this case, a receptor protein has not been identified. Although ubiquitin interacts with the Atg8 homologue LC3 in mammalian cells via non-ATG adaptor proteins (for example, SQSTM1/p62), a clear link between ubiquitination and cargo recognition is yet to be established in yeast.

SELECTIVE AUTOPHAGY IN HIGHER EUKARYOTES

Selective types of autophagy have also been well documented in higher eukaryotes. In contrast to yeast cells, there is no apparent homologue of Atg11 in mammalian cells, although analogous scaffolding proteins such as WDFY3/ALFY play a role in some types of autophagy (Filimonenko *et al.*, 2010). Furthermore, various mechanisms have emerged to confer selectivity for the autophagic degradation of different cargos, likely representing the increased complexity and diversity of mammalian cells.

In *C. elegans*, the selective degradation of PGL granules occurs through a mechanism that is comparable to the receptor-scaffold system in yeast. PGL granules are maternally derived protein aggregates that carry the germ cell determinant. PGL granules are retained in germ cells, whereas they are removed by selective autophagic degradation in somatic cells. SEPA-1, a receptor protein, directly binds the PGL granule through its interaction with the protein PGL-3 (the ligand). SEPA-1 also associates with the autophagy protein LGG-1/Atg8 via its interaction with the scaffold EPG-2 (Tian *et al.*, 2010; Zhang *et al.*, 2009). Therefore, the PGL-3-SEPA-1-EPG-2-LGG-1 protein complex forms the ligand-receptor-scaffold system for the selective autophagic degradation of the PGL granules in *C. elegans*.

In mammalian systems, the selectivity of autophagy may be mediated through ubiquitin-dependent or -independent mechanisms. In the first case, SQSTM1 (Pankiv *et al.*, 2007), CALCOCO2/NDP52 (Thurston *et al.*, 2009), OPTN (Wild *et al.*, 2011) and NBR1 (Kirkin *et al.*, 2009) have been proposed to be autophagic receptors that can link ubiquitinated cargos with the autophagy protein LC3. These proteins play important roles in the degradation of protein aggregates (aggrephagy), invading pathogens (xenophagy) and mitochondria (mitophagy), all of which have connections with pathophysiologies. For example, protein aggregates are commonly seen in various human diseases. Lewy bodies in Parkinson disease contain aggregates of DJ1 or SNCA/α-synuclein. The ubiquitination of these protein aggregates recruits SQSTM1 and leads to their subsequent autophagic degradation, although not all protein aggregates can be degraded by autophagy. During xenophagy, the autophagic degradation of invasive microbes, SQSTM1 (and other receptors), followed by LC3 (and other LC3-like proteins including members of the GABARAP subfamily), are recruited to the surface of ubiquitin-labeled bacteria (Zheng *et al.*, 2009). In PARK2/

PARKIN-mediated mitophagy, ubiquitination of several mitochondrial proteins by the E3 ubiquitin ligase PARK2 has been suggested to be important for the subsequent recognition of damaged mitochondria by the autophagic machinery. The mechanism of mitophagy is discussed in more detail later.

Ubiquitin-independent mechanisms also exist to mediate selective autophagy. For example, two mitochondrial outer membrane proteins, BNIP3L/NIX and FUNDC1, can interact with LC3, thus providing a direct association between the degrading mitochondria and the core autophagy machinery. In addition, in some cases SQSTM1 can also directly associate with cargo proteins. For example, a mutant of SOD1 (superoxide dismutase 1) that is associated with amyotrophic lateral sclerosis is targeted for autophagic degradation in a ubiquitin-independent manner through a direct interaction with SQSTM1 (Gal *et al*., 2009). Autophagy has also been demonstrated to control lipid metabolism through the selective degradation of lipid droplet triglycerides and cholesterol (Singh *et al*., 2009), although the molecular mechanism in regard to the selectivity of this process is yet to be characterized.

In summary, a variety of different mechanisms exist to mediate the selective autophagic degradation of a range of cargos in different organisms. These selective autophagic pathways share a common theme in that they all require a receptor and scaffold protein that can specifically link the degrading cargo to the autophagic machinery. The receptor proteins can interact with autophagy proteins (Atg11 and Atg8 in yeast cells, LC3 in higher eukaryotes), and they can associate with the cargo. These receptors can either bind the cargo directly through interaction with a ligand (such as occurs with Atg19, Atg36, PpAtg30, SEPA-1, BNIP3L, and FUNDC1) or they can interact with cargo that has been ubiquitinated (such as occurs with SQSTM1 and NBR1).

As already mentioned, the mitochondrion has been well documented to be a major target of autophagy. Recent research has greatly advanced our knowledge on both the molecular process and physiological significance of mitophagy from yeast to mammalian cells. Mitophagy has attracted extensive interest in the autophagy field, which is largely because of the important functions of this organelle and the well-established link between mitochondrial dysfunction and the pathogenesis of a range of diseases.

MITOCHONDRIAL DYSFUNCTION IN AGE-RELATED DISEASES

Mitochondria are essential organelles for all eukaryotes. Long known as the powerhouse of the cell, modern studies have established a central role of mitochondria in a range of different cellular activities such as calcium and iron metabolism, and apoptosis (Wallace, 2005). Conversely, reactive oxygen species (ROS), the inevitable toxic byproduct of mitochondrial oxidative phosphorylation, may cause severe damage to mitochondrial components and other parts of the cell (Wallace, 2005). Furthermore, oxidative damage caused by ROS can make mitochondria prone to additional ROS production, leading to a downward spiral that may eventually result in cell death (Miquel *et al*., 1980).

Mitochondrial dysfunction is associated with aging, diabetes, and neurodegenerative diseases. The mitochondrial theory of aging proposes that oxidative damage resulting from oxidative phosphorylation is an important contributor to the process of aging

(Miquel *et al.*, 1980). In addition, mitochondrial dysfunction is linked with the pathogenesis of several age-related neurodegenerative diseases, such as Alzheimer, Parkinson, and Huntington diseases.

Alzheimer disease (AD) is characterized by neurodegenerative changes such as cerebral atrophy. Mitochondrial mass is significantly reduced in affected cells obtained from patients with AD. Cytochrome c oxidase deficiency is often present in AD patients, which leads to an increase in ROS production and disruption of energy metabolism (Mutisya *et al.*, 1994; Zhu *et al.*, 2006). Parkinson disease (PD) is the second most common progressive disorder in the central nervous system, which is characterized primarily by the selective loss of dopaminergic neurons in the substantia nigra and the formation of intraneuronal protein aggregates (Dauer and Przedborski, 2003). Mitochondrial dysfunction plays a prevalent role in the pathogenesis of PD; a significant decrease in the activity of complex I of the electron transport chain is observed in the substantia nigra from PD patients (Mann *et al.*, 1994). In familial PD, which accounts for about 10% of all cases, several genes have been linked with the pathogenesis of PD (Dodson and Guo, 2007). Autosomal recessive mutations in PINK1 and PARK2 are associated with juvenile parkinsonism (Kitada *et al.*, 1998). Studies in *Drosophila* and primary fibroblasts derived from PD patients revealed the role of PINK1 and PARK2 in the maintenance of normal mitochondrial morphology (Yang *et al.*, 2006), suggesting that mitochondrial dynamics and function play an important role in PD pathogenesis. Huntington disease (HD) is caused by an autosomal dominant mutation in the *HTT/HUNTINGTIN* gene that affects muscle coordination and leads to a cognitive defect. Mitochondrial morphological defects and reduced activity are commonly observed in HD patients (Bossy-Wetzel *et al.*, 2008).

Considering the extensive involvement of mitochondrial dysfunction and oxidative stress in human diseases, it is essential for the cell to tightly regulate the quality, number, and activity of mitochondria – dysfunctional and superfluous mitochondria need to be eliminated in a timely manner.

MITOCHONDRIAL QUALITY CONTROL SYSTEMS

To preserve a healthy and properly functional population of mitochondria, several quality control systems that operate at different levels have been developed. First of all, mitochondria have their own proteolytic system that is responsible for the degradation of misfolded and dysfunctional mitochondrial proteins. In yeast and higher eukaryotes, ATP-dependent proteases sense the folding status of their substrates and mediate the proteolysis of misfolded proteins (Tatsuta and Langer, 2008).

In addition to proteolytic degradation of mitochondrial proteins, a recent study identified a mitochondria-to-lysosome pathway in which vesicles bud from mitochondrial tubules and sequester selected mitochondrial cargos for delivery to the lysosome for degradation. This pathway operates under normal conditions and is more active when stimulated by oxidative stress (Soubannier *et al.*, 2012).

Mitochondria can also be turned over at the organelle level by bulk degradation through autophagy. In fact, electron microscopy studies often reveal entire mitochondria within the

yeast vacuole (Takeshige *et al.*, 1992) or mammalian lysosome (Clark, 1957). Nonselective autophagy can degrade a portion of the mitochondrial population, while a selective form of autophagy, termed mitophagy, is the main pathway responsible for the degradation of damaged or superfluous mitochondria.

MITOPHAGY IN YEAST

When yeast cells are cultured in media with a nonfermentable carbon source, such as lactate or glycerol, they shift from anaerobic fermentation to aerobic respiration resulting in mitochondria proliferation. When these cells are transferred to nitrogen-starvation medium supplemented with glucose as the carbon source, mitophagy occurs primarily as a mechanism to eliminate superfluous mitochondria. In addition, mitophagy can also be induced when cells grow under aerobic respiration conditions into the post-log phase (Kanki and Klionsky, 2008). In this case, mitophagy may be induced as a quality control mechanism to eliminate damaged mitochondria caused by oxidative stress during post-log phase growth (Okamoto *et al.*, 2009).

Core autophagy machinery proteins and Atg11 are required for mitophagy, demonstrating that this is a selective form of autophagy (Kanki and Klionsky, 2008). Two genetic screens targeted to identify mitophagy-related genes led to the discovery of Atg32 as the receptor protein that confers selectivity for mitochondrial recognition and sequestration (Figure 2.1A) (Kanki *et al.*, 2009; Okamoto *et al.*, 2009).

As discussed, Atg32 is a mitochondrial outer membrane protein that contains a single transmembrane domain with its N terminus facing the cytosol and the C terminus in the intermembrane space (Kanki *et al.*, 2009; Okamoto *et al.*, 2009). As a mitochondrial resident protein, Atg32 bridges the degrading fragment of mitochondria with the autophagic machinery through its interactions with the scaffolding protein Atg11 and Atg8; however, the interaction between Atg32 and Atg8 appears not to be essential for mitophagy, because disruption of this interaction only causes a partial block of mitophagy activity (Kondo-Okamoto *et al.*, 2012). The mitochondrial reticulum is large and is not a good target for sequestration. Accordingly, fission is a critical step in the process that generates mitochondrial fragments that are amenable to inclusion within an autophagosome. Cytosolic Atg11 interacts with the fission protein Dnm1, which is subsequently recruited to mitochondria via the interaction of Atg11 with Atg32 (Mao *et al.*, 2013).

Considering the essential roles of mitochondria in the cell, the process of mitophagy needs to be tightly controlled. Several recent studies have established at least some of the regulatory mechanisms of mitophagy. First, Atg32 undergoes starvation-dependent phosphorylation (Aoki *et al.*, 2011). When mitophagy is induced, S114 and S119 on the N terminus of Atg32 are phosphorylated. These phosphorylation events are important for Atg32-Atg11 interaction and subsequent mitophagy induction. Hog1 and Pbs2, kinases of the mitogen-activated protein kinase pathway, are important for mitophagy activity, although these kinases are not directly involved in Atg32 phosphorylation (Aoki *et al.*, 2011). Instead, casein kinase 2 directly phosphorylates Atg32 on these two sites and regulates mitophagy activity (Kanki *et al.*, 2013).

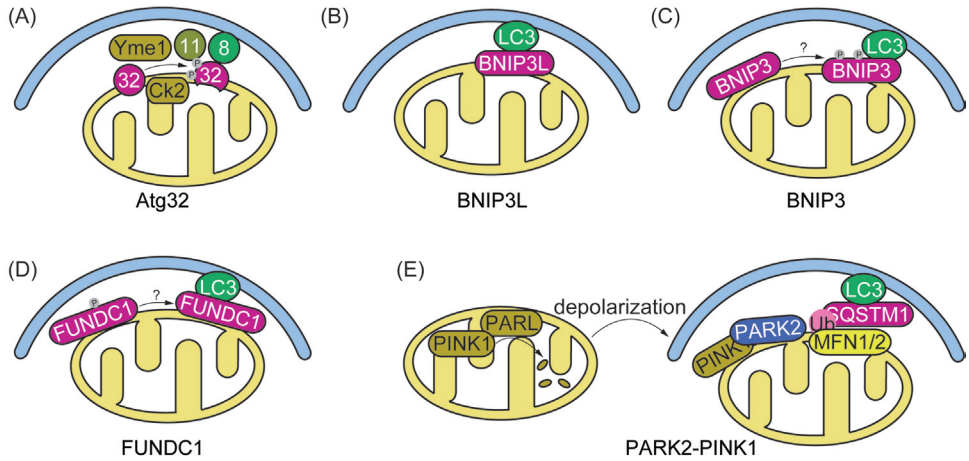

FIGURE 2.1 Different mechanisms of mitophagy are illustrated schematically (see the text for details). A. Atg32-mediated mitophagy. Atg32 is phosphorylated by casein kinase 2 (Ck2), and processed by Yme1. The modified Atg32 interacts with Atg11 and brings mitochondria to the autophagic machinery for degradation. B. BNIP3L-mediated mitophagy. BNIP3L interacts with LC3 through its LIR domain. C. BNIP3-mediated mitophagy. BNIP3 is phosphorylated by an unknown kinase. Phosphorylated BNIP3 interacts with LC3. D. FUNDC1-mediated mitophagy. FUNDC1 is constitutively phosphorylated by SRC kinase (not shown). Hypoxia leads to the inactivation of SRC and dephosphorylation of FUNDC1 by an unknown phosphatase. Dephosphorylated FUNDC1 interacts with LC3. E. PARK2-PINK1-mediated mitophagy. PINK1 is steadily imported into mitochondria, where the PARL protease mediates its degradation. Mitochondrial depolarization inhibits PARL activity and PINK1 import, which leads to the accumulation of PINK1 on the outer membrane, and the subsequent recruitment of PARK2. PARK2 then ubiquitinates several mitochondrial proteins including MFN1/2. The ubiquitinated MFN1/2 may be recognized by SQSTM1, which interacts with LC3 and directs the mitochondria to the autophagic machinery for degradation.

In addition to phosphorylation, recent work has demonstrated that Atg32 also undergoes another level of regulation through the proteolytic processing of its intermembrane space region at the C terminus (Wang *et al.*, 2013). In this case, the mitochondrial i-AAA protease Yme1 mediates Atg32 processing and is required for efficient Atg32 and Atg11 interaction and subsequent mitophagy activity. Since Yme1 is an important component of the proteolysis level mitochondrial quality control system and is responsible for the degradation of unfolded or misfolded mitochondrial proteins (Weber *et al.*, 1996), this study suggests a crosstalk between different mitochondrial quality control mechanisms.

Physiologically, mitophagy occurs in yeast as a system that acts to prevent the damaging effect of functionally compromised mitochondria (Kurihara *et al.*, 2012). When mitophagy-deficient (*atg11*Δ or *atg32*Δ) cells are challenged with nutrient starvation, mitochondria cannot be degraded and are vulnerable to damage resulting from exposure to ROS. These damaged mitochondria gradually lose their mitochondrial DNA, and cells bearing these mitochondria exhibit a "petite" phenotype, being unable to grow on nonfermentable carbon sources. Therefore, mitophagy in yeast plays an essential role in maintaining mitochondrial quality.

MITOPHAGY IN MAMMALIAN CELLS

Autophagy also plays a critical role in mitochondrial degradation in higher eukaryotes. In fact, mitochondria were detected inside an autophagosome in the kidney cells of mice as early as the 1950s (Clark, 1957). Multiple stress conditions can induce mitophagy in mammalian cells such as starvation (Egan *et al.*, 2011), CCCP-induced disruption of mitochondrial membrane potential (Narendra *et al.*, 2008), and hypoxia (Liu *et al.*, 2012). In addition, mitophagy can also be observed during normal development such as occurs during the maturation of erythrocytes (Sandoval *et al.*, 2008) and in the process of spermatogenesis in some organisms (Al Rawi *et al.*, 2011). Our knowledge of the molecular process of mitophagy in mammalian cells has greatly expanded during recent years. As mentioned above, there is no clear counterpart of Atg11 that acts as a scaffold in mammalian cells so that the molecular machinery involved in mammalian mitophagy is more diverse. In fact, in contrast to yeast cells, most mammalian cells need to constantly undergo aerobic respiration, so it is possible that mammalian cells may face a more stringent and complicated situation with regard to the regulation of mitochondrial quantity and quality. During recent years, different proteins have been discovered to mediate mitophagy, either in different cellular processes or in response to different stimuli. Despite the differences in the particular mechanisms, the association of mitochondria with the molecular machinery of autophagy typically involves LC3.

BNIP3L and BNIP3

One of the very first studies in mammalian cells in regard to the molecular mechanism of mitophagy is the discovery of BNIP3L in the selective elimination of mitochondria during the maturation of erythrocytes (Sandoval *et al.*, 2008). During terminal differentiation, erythroid cells undergo autophagic elimination of organelles including mitochondria. BNIP3L, a BH3-only member of the BCL2 family, is a mitochondrial outer membrane protein essential for this process (Schweers *et al.*, 2007). *bnip3l*$^{-/-}$ mice display a reduced number of mature erythrocytes, and exhibit mitochondrial retention and defective entry of mitochondria into autophagosomes (Sandoval *et al.*, 2008). This study also established the primary link between the loss of mitochondrial membrane potential and mitophagy. More recent studies revealed that BNIP3L has a WXXL motif in its N terminus and can bind to Atg8 homologues, especially LC3A and the GABARAP subfamily. The interaction of BNIP3L with Atg8 homologues is upregulated upon mitochondrial stress such as occurs following treatment with rotenone or the mitochondrial uncoupler CCCP, which potently induce mitophagy. Mutation of the WXXL motif reduces the interaction and causes a defect in mitochondrial clearance (Novak *et al.*, 2010). Therefore, BNIP3L plays a role in mitophagy through linking the degrading mitochondria with LC3. The BNIP3L-LC3 model is reminiscent of the Atg32-Atg11 model in yeast cells, and thus BNIP3L is also considered to function as a mammalian mitophagy receptor (Figure 2.1B).

More recently, BNIP3, a protein homologous to BNIP3L, has been reported to be involved in mitophagy (Zhu *et al.*, 2013). BNIP3 shares high sequence similarity with BNIP3L including the LIR motif, and BNIP3 also interacts with LC3. Both proteins also have a similar mitochondrial localization pattern. Interestingly, serine residues 17 and 24 flanking the BNIP3

LIR region are phosphorylated, and this phosphorylation is important for binding to the LC3 family proteins LC3B and GABARAPL2/GATE-16 (Zhu *et al.*, 2013). This result suggests that a similar regulatory mechanism of receptor phosphorylation as with Atg32 may also occur with mitophagy receptor proteins in mammalian cells (Figure 2.1C). However, whether BNIP3L undergoes a similar type of regulation through phosphorylation remains to be discovered.

FUNDC1

Another mitochondrial outer membrane protein, FUNDC1, functions as a mitophagy receptor and plays an important role in hypoxia-induced mitophagy (Liu *et al.*, 2012). FUNDC1 interacts with LC3 through an LIR consisting of the residues YXXL (equivalent to WXXL), and mutation of this region abolishes its interaction with LC3 and subsequent mitophagy. FUNDC1 overexpression induces mitophagy in multiple cell lines and knockdown of endogenous FUNDC1 causes a defect in hypoxia-induced mitophagy. Interestingly, FUNDC1 seems to play a less important role in starvation-induced mitophagy or CCCP-induced mitophagy, suggesting different mechanisms may be responsible for mitophagy that occurs in response to different stress conditions.

Similar to the case of Atg32 and BNIP3L, FUNDC1-mediated mitophagy is also regulated by its phosphorylation. SRC kinase phosphorylates FUNDC1 at Tyr18 in the LIR motif under normal physiological conditions. In contrast to the regulation of BNIP3L and Atg32, SRC is inactivated upon hypoxia, and FUNDC1 is subsequently dephosphorylated, leading to its enhanced association with LC3, which in turn results in the selective incorporation of the degrading mitochondria into the phagophore. The phosphatases that are predicted to be involved in the dephosphorylation of Tyr8 on FUNDC1 have not been identified, but this would add another level of regulation (Figure 2.1D).

In conclusion, the LIR-containing motif in BNIP3L and FUNDC1, functionally similar to the Atg8-family interacting motif in Atg32, acts as a central mitophagy signaling transduction domain and is thus highly regulated in response to different cellular signals.

PARK2 and PINK1

The discovery of the involvement of PARK2 in mitophagy is another milestone in mammalian mitophagy studies. PARK2 is a cytosolic E3-ubiquitin ligase that mediates proteasomal protein degradation (Kitada *et al.*, 1998). Mutations in *PARK2* are associated with the familial autosomal recessive juvenile form of Parkinson disease (Kitada *et al.*, 1998), and the association of PARK2 with mitophagy provides an intriguing possibility to explain the pathogenesis of PD. Upon treatment with the mitochondrial uncoupler CCCP, PARK2 rapidly translocates from the cytosol to mitochondria, followed by substantial mitochondrial degradation. PARK2 is selectively recruited to the mitochondrial fragments with reduced membrane potential, suggesting that the cell has a mechanism to distinguish between healthy and damaged mitochondria; recruitment of PARK2 specifically promotes the elimination of dysfunctional mitochondrial fragments. The degradation of damaged mitochondria is dependent on autophagy proteins such as ATG5, demonstrating that the process

is mediated through autophagy. Indeed, these PARK2-marked mitochondrial fragments are LC3-positive, providing direct evidence that the clearance of these fragments occurs through autophagy. In addition, similar PARK2 translocation is also observed following treatment with paraquat, a herbicide frequently used in the study of PD.

The translocation of PARK2 to the damaged mitochondria requires another PD-related protein, PINK1 (PTEN induced putative kinase 1). PINK1 is a mitochondrial membrane-anchored protein kinase. Genetic studies in *Drosophila* suggested that Pink1 and Park function in the same pathway. Several studies reported that PINK1 plays a role in the recruitment of PARK2 (Matsuda *et al*., 2010; Narendra *et al*., 2010b; Vives-Bauza *et al*., 2010), and overexpression of PINK1 is sufficient to induce PARK2 translocation to mitochondria even without mitochondrial membrane depolarization via uncouplers. In healthy mitochondria, PINK1 is constitutively expressed and imported via the TOMM-TIMM complex, to the mitochondrial inner membrane, where it is rapidly degraded through proteolysis by the inner membrane rhomboid protease PARL (presenilin associated, rhomboid-like) (Zhu *et al*., 2013). However, when mitochondria depolarize, PINK1 import into the inner membrane is impaired and subsequent proteolysis is inhibited, leading to a rapid accumulation of PINK1 on the outer membrane of damaged mitochondria, and the subsequent recruitment of PARK2. While PINK1 has been reported to be able to bind and phosphorylate PARK2, and PINK1 kinase activity is also required for PARK2 recruitment (Kim *et al*., 2008), how PINK1 phosphorylation of PARK2 affects the latter's recruitment and subsequently drives mitophagy is still under investigation.

As an E3 ubiquitin ligase, PARK2 ubiquitinates a subset of mitochondrial substrates (Chan *et al*., 2011). As with PINK1 function, however, how PARK2-mediated ubiquitination initiates the onset of mitophagy remains unclear. Multiple mitochondrial outer membrane proteins are rapidly degraded following PARK2-mediated polyubiquitination, suggesting the activation of the ubiquitin-proteasome system is an important step prior to PARK2-mediated mitophagy (Chan *et al*., 2011). VDAC1 is a substrate for PARK2 and may be required for PARK2-mediated mitophagy (Geisler *et al*., 2010), but this conclusion is not fully supported by other studies (Narendra *et al*., 2010a). MFN1 and MFN2 are also ubiquitinated by PARK2 upon mitophagy induction and are subsequently degraded in the proteasome (Tanaka *et al*., 2010). MFN1 and MFN2 are large GTPases that mediate mitochondrial fusion. Therefore, the degradation of MFN1 and MFN2 results in mitochondrial fragmentation, which promotes subsequent mitophagy; this process may be functionally equivalent to Dnm1-dependent fission and mitophagy in yeast. However, PARK2 is also able to ubiquitinate the mitochondrial fission protein DNM1L/DRP1, leading to its degradation (Wang *et al*., 2011). Furthermore, what mediates the association of degrading mitochondria marked by PARK2 with the autophagic machinery remains unclear. The ubiquitin binding adaptor protein SQSTM1 may play such a role due to its ability to bind to both ubiquitin and LC3; and SQSTM1 accumulates on mitochondria following PARK2-mediated ubiquitination (Geisler *et al*., 2010). However, whether SQSTM1 is essential for PARK2-mediated mitophagy remains controversial (Narendra *et al*., 2010a). Nonetheless, these lines of research provided important insights into how mitochondrial dysfunction may be associated with the pathogenesis of PD. Indeed, several studies showed that disease-associated *PARK2* and *PINK1* mutations result in defective mitophagy, thereby implicating the

involvement of mitophagy defects in the development of PD (Lee *et al*., 2010; Matsuda *et al*., 2010; Narendra *et al*., 2010b) (Figure 2.1E).

DISCUSSION AND PERSPECTIVES

Although our understanding of the molecular process and physiological significance of mitophagy has greatly advanced during the last few years, further questions remain to be addressed. First, despite the well-established model of PARK2-mediated mitophagy, the research has primarily been carried out using mammalian cell lines. Gaps are apparent in our understanding of the activity of mitophagy and the pathogenesis of age-related diseases such as PD, AD, and HD. Although PARK2 and PINK1 mutation seen in PD patients result in extensive mitophagy defects in cultured cells, *in vivo* evidence is still lacking in regard to the connection between defective mitophagy and the onset of PD. It will therefore be very helpful to generate knockout or knockdown mouse models of the mitophagy-related proteins to study their relationship with distinct diseases.

Second, the list of mitophagy-related proteins is expanding; however, we are still largely ignorant of the upstream signaling events that regulate mitophagy. Unidentified kinases, phosphatases and other signaling molecules play important roles in the regulation of mitophagy through modification of the relevant receptor proteins. How these signaling molecules are associated with the bigger picture of mitochondrial metabolism, such as ROS production and energy generation, is a question that needs to be further addressed.

Third, the discovery of different mechanisms of mitophagy provokes the intriguing question of the possible cross-talk between these mitophagy pathways. Evidence suggests that these pathways are not totally independent of each other. For example, BNIP3L was initially identified in the process of erythrocyte maturation; however, later studies demonstrated that it is also involved in CCCP-induced mitophagy (Novak *et al*., 2010), where PARK2 is known to play a key role. How cells coordinate these different mitophagy pathways remains an interesting topic for future study.

Fourth, the discrepancy between yeast mitophagy and mammalian mitophagy needs more attention. Mitochondrial dysfunction caused by a loss of mitochondrial membrane potential has been clearly shown to be a potential inducing signal for mitophagy in mammalian cells; however, the same signal does not induce mitophagy efficiently in yeast (our unpublished data). Nonetheless, mitophagy is important for yeast to survive extensive oxidative stress. Intriguingly, the yeast *Saccharomyces cerevisiae* is adapted to carry out fermentation, instead of respiration, during normal growth conditions in the presence of glucose regardless of the availability of oxygen, which is reminiscent of the metabolic pattern of tumors. Therefore, a deeper understanding and characterization of the regulation of mitophagy in yeast may provide insight into the connection between mitophagy and cancer.

While mitochondrial activity and quality are highly regulated through mitophagy-dependent degradation, recent studies in yeast demonstrated that the maintenance of a healthy population of mitochondria is in return required for proper autophagy activity (Graef and Nunnari, 2011). Defects in mitochondrial respiration cause the activation of cAMP-dependent protein kinase A and lead to autophagy inhibition. Therefore, this observation adds another level of interplay between mitochondrial function and autophagy, and

suggests a possible link between mitochondrial dysfunction and disease through the deregulation of autophagy. Since this study was carried out in yeast cells, it will be intriguing to see whether the same mechanism is also present in higher eukaryotes.

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CHAPTER

3

Principles of Mitophagy and Beyond

Aviva M. Tolkovsky and Kim Tieu

OUTLINE

Abstract

In eukaryotic cells, mitochondria express tags that enable them to be selectively engulfed by the autophagic machinery and delivered to the lysosome for degradation. This process is termed *mitophagy*. This review highlights the tremendous progress made in delineating the principles of mitophagy in yeast and metazoans and discusses some of the roles of mitophagy in cell homeostasis.

INTRODUCTION

Mitophagy is the process whereby mitochondria are specifically engulfed by autophagosomes and targeted for degradation in the lysosome. This process is distinct from general macroautophagy in that in the latter process, mitochondria are engulfed by autophagosomes nonspecifically together with other cytoplasmic organelles, for example in livers

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of rats treated with glucagon (Ashford and Porter, 1962). Clearly, for proper execution of mitophagy, mitochondria need to display a specific address or tag that is activated/modified so that the mitochondria destined for autophagy are distinguished from their cohorts that are not. Indeed, specific mitochondrial tags have now been identified in both yeast and metzoans, and the mechanisms of mitophagy have thereby been greatly clarified. Still lagging behind is the physiological and pathological relevance of mitophagy, given that autophagy can also degrade mitochondria nonselectively, and there are other pathways that can remove mitochondria, or selective mitochondrial components, via intrinsic proteases, proteasomes, or lysosomes (Soubannier *et al.*, 2012; Vincow *et al.*, 2013). Although the protein Atg32, the mitochondrial mitophagic tag in yeast, has no overall sequence homology to the mammalian tag proteins Pink1, Fundc1, NIX, and BNIP3, there are compelling mechanistic similarities that demonstrate common principles underlying mitophagy in all eukaryotic cells. In this review we describe current understanding of these principles in yeast and metzoans and delve into possible roles of mitophagy in cell survival, development, and adaptation.

YEAST MITOPHAGY

Principles and Mechanisms

Mitophagy plays a central role in the physiology of the Saccharomycetaceae family of yeast. In *S. cerevisiae*, growth in nonfermentable substrates, such as lactate or glycerol, is dependent on mitochondrial respiration. Under these conditions there is no evident mitophagy, even during nitrogen starvation, which robustly activates general macroautophagy (henceforth referred to as autophagy). However, fewer mitochondria are required during the post-log maintenance phase (henceforth referred to as stationary), or upon transfer from a nonfermentable growth medium to a fermentable (glucose) medium. During growth in glucose medium, mitochondria can be diluted during cell division, but upon nitrogen starvation (henceforth referred to as starvation), cell growth is diminished and this is when mitophagy is activated. These responses maximize coupling between nutrient utilization and energy production, so it is not surprising that divergent yeast genera in the family Saccharomycetaceae utilize the same strategies to enact mitophagy (Farre *et al.*, 2013; Kanki *et al.*, 2009; Okamoto *et al.*, 2009b). However, in organisms that cannot ferment their fuel, such as humans, mitochondria are always essential for survival, even in cancer cells undergoing vigorous aerobic glycolysis (Koppenol *et al.*, 2011; Wallace, 2005). Hence, mitophagy is more likely to be linked to disturbances in mitochondrial homeostasis, as is the case when yeast mitochondria are damaged during respiratory growth.

For ease of description, mitophagy is split into steps, summarized schematically in Figure 3.1:

1. Phosphorylated Atg32 is the mitochondrial tag for mitophagy. In *S. cerevisiae*, Atg32 is a single pass transmembrane protein of ~60 kDa that is stably expressed in the outer mitochondrial membrane. Its N-terminus faces the cytoplasm and its C-terminus faces the intermembrane space (IMS) (Okamoto *et al.*, 2009b). Upon a switch to conditions (such as starvation) that induce mitophagy, MAPK signaling pathways are induced that

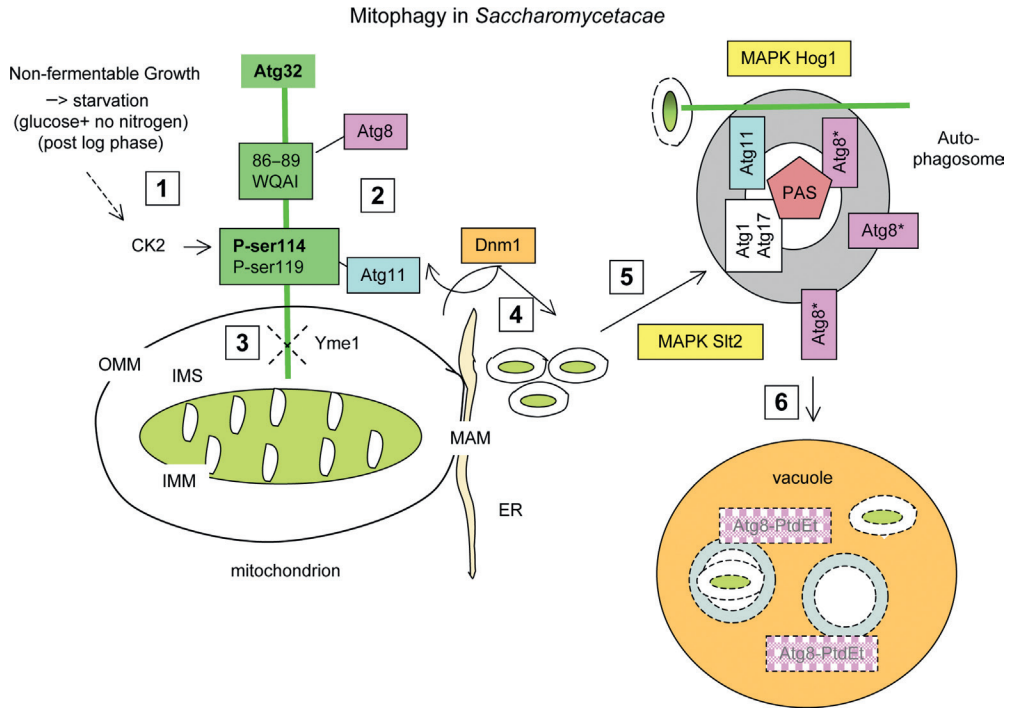

FIGURE 3.1 Suggested steps in yeast mitophagy: 1. Atg32 phosphorylation; 2. Recruitment of Atg11 to phosphoserines and Atg8 to the AIM/LIR motif on Atg32; 3. Proteolytic digestion of the IMS (intermembrane space) C-terminus of Atg32; 4. Recruitment of Dnm1 to Atg11 and mitochondrial fragmentation at the MAM (mitochondria-associated ER membrane); 5. Recruitment of Atg32-bound sorted mitochondrial fragments to the PAS (pre-autophagosomal structure); 6. Fusion with the vacuole (lysosome) and degradation. Abbreviations: CK2, casein kinase 2; OMM and IMM, outer and inner mitochondrial membranes; ER, endoplasmic reticulum; Atg8*, lipidated Atg8.

culminate in Slt1 and Hog1 activation, yeast homologues of stress-activated MAPKs (Aoki *et al.*, 2011; Mao *et al.*, 2011). However, these kinases do not phosphorylate Atg32 but, rather, participate later in the mitophagic process (see step 5). Surprisingly, though, given its high basal activity, casein kinase 2 (CK2) seems to be essential for Atg32 activation, capable of directly phosphorylating Atg32 *ex vivo* (Kanki *et al.*, 2013). How it is activated when mitophagy is signaled and whether it is the true and only Atg32 kinase are still not clear.

2. A tripartite Atg32/Atg11/Atg8 initiator complex is formed: Phosphorylation of Atg32 at serines 114/119 is required for recruiting the scaffold protein Atg11, which then recruits core autophagy proteins to the site of autophagosome biogenesis, known as the *preautophagosomal structure* or *phagosome assembly site* (PAS). Unique to yeast, the PAS lies in close association with the vacuole (lysosome), facilitating close contact to the site of autophagosome/lysosome fusion. Notably, Atg11 is also required for other selective types of autophagy (pexophagy, cytoplasm to vacuole targeting (Cvt), piecemeal autophagy of the nucleus) (Suzuki, 2013). Hence the uniqueness of mitophagy lies in

its exclusive receptor, Atg32. Concomitantly, but independently of Atg11 binding, Atg8, the MAP-LC3 family yeast homologue, is recruited to Atg32's WQAI motif, known as the Atg8-family interacting motif (AIM) (equivalent to the LIR, LC3-interacting region (Birgisdottir *et al.*, 2013; Noda *et al.*, 2010)) (Okamoto *et al.*, 2009b). Atg8 is essential for autophagosome assembly. For a hypothetical model of how the three proteins interact at the molecular level, see Farre *et al.* (2013). Interestingly, Atg8 binding to Atg32 is reported to occur independently of its lipid conjugation with phosphatidyl-ethanolamine (PtdEth), although the latter is a prerequisite for Atg8's insertion into the autophagosome membrane and for subsequent autophagosome growth and maturation (Kondo-Okamoto *et al.*, 2012). Perhaps lipid conjugation is not necessary but is still preferred for efficient complex formation.

3. Additional modifications to Atg32 may enhance its specificity: Phosphorylation of Atg32 is necessary for its recognition by Atg8/Atg11 but is it sufficient? Analysis of Atg32 showed that the C-terminal IMS portion of Atg32 is not necessary for directing organelle-specific autophagy since Atg32 expression of the N-terminus (with a transmembrane sequence targeted to peroxisomes) was sufficient to recruit peroxisomes for pexophagy (Kondo-Okamoto *et al.*, 2012). Aoki *et al.* (2011) reported that when mitophagy is stimulated in atg11Δ cells (so that activated Atg32 can accumulate without it being degraded), the molecular mass of Atg32 decreases before it increases due to its phosphorylation, but the reason for this was not addressed. Recent work explains this finding by showing that upon induction of mitophagy, the C-terminus of Atg32 is cleaved by the intermembrane mitochondrial quality control (iAAA) protease Yme1 (Wang, K. *et al.*, 2013). Deletion of Yme1 inhibited mitophagy, suggesting that it is essential, but at later times there seems to be normal mitophagy in Yme1Δ cells (Welter *et al.*, 2013), which fits with the idea that an early, specific form of mitophagy gives way to other mitochondrial degradation pathways involving Yme1, some of which may still involve the vacuole (Campbell and Thorsness, 1998). A transient increase in Atg32 abundance prior to onset of delivery to the vacuole was also noted (Okamoto *et al.*, 2009a) but although the amount of Atg32 can be rate limiting under certain conditions, the increase was not necessary for mitophagy to occur (Eiyama *et al.*, 2013).
4. Mitochondrial fragmentation facilitates recruitment to the PAS: Deletion of genes that promote fission of mitochondria reduces starvation or post-log mitophagy (Abeliovich *et al.*, 2013; Mao *et al.*, 2013). A key initiator of this event is Dnm1 (Dynamin-related GTPase 1, which is required for mitochondrial fission) though not when a weaker, more delayed form of mitophagy is induced by rapamycin (Mendl *et al.*, 2011). Although Fis1 is the mitochondrial receptor for Dnm1 (Mozdy *et al.*, 2000) it may not always be required (Nieto-Jacobo *et al.*, 2012), perhaps because Dnm1 binds to Atg11 instead. Indeed, visualization of Atg11/Atg32 binding via bimolecular fluorescence complementation showed that upon induction of mitophagy, Atg11/Atg32 complexes form foci that recruit Dnm1, labeling the region where fragmentation ensues (Mao and Klionsky, 2013). ER may also participate in mitophagy, in line with findings that mitochondrial fragmentation occurs at ER/mitochondria contact sites (Friedman *et al.*, 2011; Michel and Kornmann, 2012), possibly via the ER–mitochondria encounter structure (ERMES), also known as the mitochondria-associated ER membrane (MAM). Indeed, it has been proposed that

the ERMES/MAM is the donor membrane for autophagosome biogenesis in mammalian cells (Lamb *et al.*, 2013).

Why is mitochondrial fragmentation important for mitophagy? In mammalian cells, fragmentation may be important for segregating defective depolarized fragments from healthy counterparts (Twig *et al.*, 2008) but in yeast, during starvation-induced mitophagy the mitochondria appear to remain polarized. It has been proposed that fragmentation is required to convert large tubular mitochondria into phagocytosable sizes (Mao *et al.*, 2013), but Abeliovich *et al.* (2013) suggest that dynamic mitochondrial turnover is required, rather than a smaller size, whose purpose is the segregation of select mitochondrial matrix proteins into subparticles that do not get delivered to the vacuole during mitophagy of otherwise whole mitochondria. Submitochondrial particle segregation is an emerging concept in mitophagy research, and fits with observations in mammalian cells that mitochondrial outer membrane proteins may be degraded via the proteasome, and not via mitophagy, when mitochondria are damaged, as will be discussed further.

5. Because MAPK cascades link extracellular signals to intracellular phosphorylation events, the stress-activated protein kinases Slt1 and Hog1 (and respective upstream kinases in their cascades) have been conceptualized as being upstream of Atg32 phosphorylation. However, this is not their role. Rather, Slt1 facilitates recruitment of mitochondrial fragments to the PAS (Mao and Klionsky, 2011), just as it aids positioning of other organelles undergoing specific autophagy, such as peroxisomes during pexophagy. Interestingly, Hog1 is required only for mitophagy, but participates in mitophagy even later than Slt1, since Atg32 is already at the PAS in Atg1ΔHog1Δ mutants. Hog1 connects osmoregulation to nitrogen-starvation-induced (but not rapamycin-induced) autophagy (Prick *et al.*, 2006) but the reason why it is activated specifically during mitophagy is not known. Perhaps it is an adaptive response to increased osmotic pressure during cell shrinkage (Prick *et al.*, 2006).
6. Completion. At the PAS, several core autophagy genes, such as Atg1, Atg17, Atg29, and Atg31, contribute to completion of autophagosome formation and fusion/delivery to the vacuole (Eiyama *et al.*, 2013).

In conclusion, mitophagic stimuli transform Atg32 from being a uniformly distributed OMM protein into a specific tag via its phosphorylation, cleavage, and increased abundance. These changes are not dissimilar to the changes that occur to the mitochondrial tags in higher eukaryotes with the notable exception of absence of an ubiquitination-dependent bridging protein, Atg11, and its direct association with the PAS perhaps fulfiling this role in yeast. Ubiquitination linked to proteasome function features prominently in yeast mitochondrial fusion (Bertolin *et al.*, 2013; Cohen *et al.*, 2011) so a connection with ubiquitylation and mitophagy might still be revealed. Following on from this event, complex signaling and recruitment events occur at the PAS, some of which are specific to mitophagy, though their roles are still not clear. Fragmentation or fission/fusion cycles of the mitochondrial network are also important, in line with processes described in higher eukaryotes. Segregation of certain mitochondrial components prior to mitophagy is beginning to be featured in studies of higher eukaryotes, but many of these processes may deviate from mitophagy *per se*. A major enigma in yeast studies is how mitochondrial segregation of components takes place prior to mitophagy.

The Importance of Mitophagy in Yeast

As pointed out above, the fate of mitochondria and mitochondrial proteins will depend in part on the conditions under which mitophagy is studied. Even under conditions where the core autophagic machinery is inhibited so that Atg32-tagged mitochondria would be expected to accumulate (Atg1Δ or Atg7Δ, for example), Atg32 is eventually degraded during either the post log stationary phase or starvation (Okamoto *et al.*, 2009b; Wang, K. *et al.*, 2013), indicating that there are autophagy-dependent and independent mechanisms of Atg32 (and mitochondrial?) degradation pathways outside of mitophagy. Identification of which process is occurring is crucial for correct interpretation of results, especially when trying to understand the connection between mitophagy and cell fate.

Given the availability of Atg32Δ mutants, it has become possible to address the question of whether mitochondrial damage provokes mitophagy in yeast. Deffieu *et al.* (2013) deliberately inhibited the function of respiratory chain components in yeast growing under respiratory (lactate) conditions. Interestingly, Antimycin A (a bc1 complex III inhibitor) and KCN (complex IV cytochrome oxidase inhibitor) appeared to induce general autophagy that was nevertheless dependent on Atg32 and Atg11 and on the Slt1 MAPKK Bck1. In keeping with this observation, no delivery of mitochondrial marker proteins to the vacuole was detected and mitochondrial degradation was not diminished in a dnm1Δ mutant. However, general autophagy is well maintained in Atg32Δ or Atg11Δ mutants during stationary phase or starvation (Kanki *et al.*, 2009; Okamoto *et al.*, 2009b) so why autophagy in this particular setting depends on these genes is unclear. Assuming mitophagy did not happen at later time points, it seems that mitophagy is not simply produced by reduced respiration or increased ROS production, both of which were induced by these inhibitors. A role for ROS in signaling mitophagy was suggested (Okamoto *et al.*, 2009b) by showing that N-acetyl-cysteine (NAC) prevents Atg32-dependent mitophagy under respiratory conditions. However, NAC may be providing cysteine for production of glutathione (Deffieu *et al.*, 2009) rather than acting as a general antioxidant, since no other antioxidants prevented mitophagy.

Interestingly, other inhibitors of the respiratory chain (myxothiazol, a complex III inhibitor acting at a different site; oligomycin, a F0F1 ATP synthase inhibitor that also prevents production of ATP by reverse activity of the ATPase; CCCP, uncoupler and dissipator of mitochondrial membrane potential used extensively in mitophagy studies in mammalian systems) did not induce either autophagy or mitophagy. The authors attribute this distinction to a specific reduction in cytochrome b that only occurs with antimycin A or KCN. Perhaps there are "guardians of mitochondria" that prevent mitophagy under respiratory growth at an early stage, whereas general autophagy is needed for cell viability. However, another study showed that during nitrogen starvation, respiration-deficient cells lacking cytochrome c1, a component of the cytochrome bc1 complex, or Cox7, a subunit of cytochrome c oxidase, or cells treated with antimycin A, still activated canonical Atg32/Atg11 dependent mitophagy – although general autophagy was activated first, the two occurring concomitantly (Eiyama *et al.*, 2013). Eiyama and co-workers also noted that the amount of mitophagy varied greatly depending on growth and substrate conditions, which may explain the lack of mitophagy reported by Deffieu *et al.* (2013).

What about the role of mitophagy in cell survival? It has been thought that mitophagy is beneficial for maintenance of cell survival when mitochondria are damaged, but recent

work shows that this rule does not apply under all conditions. Richard *et al.* (2013) investigated the effects of Atg32Δ on chronological life span (CLS; how long cells survive once they deplete glucose and start depending on ethanol generated during fermentation). Normally, limiting glucose to 0.2% causes caloric restriction that prolongs CLS when other nutrients are abundant. However, Atg32Δ cells undergoing glucose-limited caloric restriction showed several mitochondrial aberrations, and ROS-induced damage to proteins, lipids, and mtDNA, that shortened mean and maximum CLS. However, a note of caution is warranted since Hughes and Gottschling (2012) found that in replicative aging (where the test is how many times a mother cell can produce a daughter cell), loss of longevity of the mother cells was simply due to progressive loss of vacuolar acidity and once this was restored, life span was not limited. Whether mother cells would be rescued in Atg32Δ cells, and whether there are changes in pH that limit life span in the CLS model is of interest.

Several investigators have found that it is autophagy rather than mitophagy that is important for cell survival, although defective mitochondria do accumulate in Atg32Δ and Atg11Δ cells. Suzuki *et al.* (2011) found that growth and survival of Atg1Δ and other core autophagy mutant cells was limited in nitrogen starvation medium (having grown previously in the presence of glucose). These autophagy-deficient cells accumulated respiratory and mtDNA-deficient mitochondria but rather than exacerbating the pathology, Atg32Δ or Atg11Δ mutants were protected, suggesting that mitophagy was detrimental. Kurihara *et al.* (2012) also found that transfer of Atg32Δ or Atg11Δ mutants from lactate to starvation medium maintained living, though small, colonies, albeit with reduced mtDNA content and more ROS production. However, the lack of autophagy (Atg1Δ) killed the cells. Finally, alpha synuclein (SCNA), whose misfolding and aggregation is associated with Parkinson's disease, was expressed in *S. cerevisiae* (Sampaio-Marques *et al.*, 2012). In a fermenting (glucose) medium, expression of wt SCNA or a toxic mutant (A53T) increased Atg32 mRNA and mitophagy (as well as Atg8 mRNA and autophagy) yet reduced CLS, which was restored in ATG11Δ or ATG32Δ mutants. Interestingly, Atg32-mediated mitophagy was mediated via a novel player in mitophagy research, the yeast sirtuin-1 homologue Sir2. Suzuki (2013) proposes that under nitrogen starvation, autophagy-deficient cells lose the ability to synthesize replacement respiratory chain components important for mitochondrial biogenesis and ROS-scavenging enzymes. Perhaps mitophagy becomes detrimental because it removes those mitochondria that do succeed in replacing some of the mitochondrial components or, as suggested by Sampaio-Marques *et al.* (2012), mitophagy in SCNA-expressing aged cells may become less specific, removing nonmitochondrial components required for cell viability. Taken together, under starvation or stationary conditions nonselective autophagy, rather than mitophagy, plays an essential role in preventing ROS accumulation, and thus in maintaining mitochondrial function. The failure to respond to starvation is the major cause of cell death in ATG mutants (Suzuki *et al.*, 2011).

MITOPHAGY IN HIGHER EUKARYOTES

In metazoans, most of what we know about control of mitophagy is due to studies in *Drosophila* and mammalian cells; there is some work in *C. elegans* that is mainly related to the fate of paternal mitochondria from sperm after fertilization. Distinct from yeast, higher

eukaryotes do not have a single vacuole (although this can fragment under some conditions in yeast), nor is there a single PAS. Rather, cells produce several pre-autophagosomal compartments, marked by proteins such as Atg14L, several autophagosomes, and can have several endo/lysosomes whose abundance can be independently regulated; an increase in their abundance may enhance the rate of mitophagy simply through increased likelihood of fusion. Recent work has identified the SNARE protein Syntaxin 17 (STX17) as being necessary for fusion of autophagosomes and lysosomes (Itakura *et al.*, 2012), and for forming autophagosomes at the ER–mitochondria contact sites in mammalian cells (Hamasaki *et al.*, 2013) and in *Drosophila* (Takats and Juhasz, 2013). These steps will not be addressed further in this review.

Ubiquitination of targets destined for mitophagy is another central motif that only appears thus far in metazoans. Besides the tag itself, ubiquitination of mitochondrial proteins is important in specifying mitochondria for degradation and for recruiting bridging proteins such as p62/sequestosome1 (SQSTM1) to the mitochondrial surface. Uniquely, p62 family members express a UBA motif, which binds to ubiquitinated proteins, a multimerization motif (PB1) that promotes clustering, and an LIR motif which binds lipidated LC3-family proteins (e.g., LC3-II) embedded in the autophagosome membrane (Birgisdottir *et al.*, 2013; Kirkin *et al.*, 2009). Ubiquitination of specific proteins may become an even more important motif as novel pathways for delivery of mitochondrial proteins to the lysosome for degradation become evident (Soubannier *et al.*, 2012; Vincow *et al.*, 2013).

Mitochondrial fragmentation is also commonly observed in mitophagy and it has been suggested that fragmentation is important for segregating dysfunctional mitochondria from healthy counterparts before their mitophagy (Twig *et al.*, 2008). To test this more rigorously, Gomes and Scorrano (2011) examined mitochondria in cells with defects in components of the fusion/fission machinery during starvation. They show that starvation induces mitochondrial elongation, which spares them from mitophagy (during autophagy), and this is accompanied by increased mitochondrial function. Similar mitochondrial elongation occurs in liver and muscle, organs prone to autophagy during starvation. This effect does not depend on autophagy itself, but provides an important mechanism to counterbalance mitochondrial elimination during autophagy.

In the following sections we review the roles of three tags that have been identified in higher eukaryotes: Fundc1, NIX (also named BNIP3L)/BNIP3, and Pink1.

Fundc1

Least is known about Fundc1 (FUN14 domain-containing protein 1). Fundc1 is an abundant outer mitochondrial membrane protein of ~17kDa that is well conserved between higher metzoans (Liu, L. *et al.*, 2012a). It is organized like Atg32, the N-terminus facing the cytosol and the C-terminus projecting into the IMS. Hypoxia induces Fundc1-dependent mitophagic flux and Fundc1 knockdown prevents hypoxia-induced mitophagy in both human and murine cell lines without altering starvation-induced autophagy. Fundc1-dependent mitophagy requires its LIR motif, YVEL, through which it binds to LC3-II. Fundc1 is suggested to convert into a tag via hypoxia-induced dephosphorylation; under normoxia, Fundc1 is phosphorylated on tyrosine 18 of the LIR motif YVEL, thereby abrogating the binding of Fundc1 to LC3-II. Although mitochondrial fragmentation also

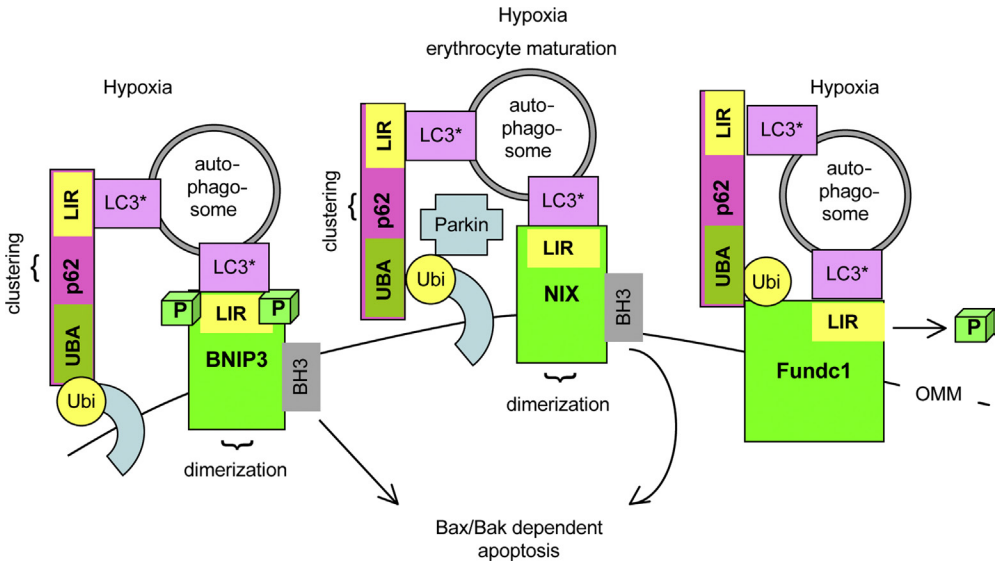

FIGURE 3.2 Suggested interactions between BNIP3, NIX, and Fundc1, with LC3 and the UBA/LIR domains of p62. Note that the involvement of p62 and mitochondrial protein ubiquitination in BNIP3-mediated mitophagy is hypothetical, whereas Parkin has been implicated in NIX-mediated mitophagy after membrane potential collapse by CCCP. Other modifications include ubiquitination of mitochondrial membrane proteins or Fundc1, phosphorylation of BNIP3, dephosphorylation of Fundc1, and dimerization of BNIP3 and NIX. p62 also has a PB1 motif (not depicted) through which it may promote clustering of mitochondrial fragments prior to mitophagy. Abbreviation: LC3*, lipidated LC3.

accompanies Fundc1-dependent mitophagy, fragmentation still occurs in an LIR mutant but this does not mean that fragmentation is not dependent on Fundc1. Recently, Fundc1 was reported to be ubiquitinated by the E3 ubiquitin ligase Parkin (Sarraf *et al.*, 2013) (see section on Pink1 following) and thus may be a binding partner of p62/SQMT1 in addition to its binding to LC3-II. A summary of these interactions is depicted in Figure 3.2. Many questions remain: is Fundc1-dependent mitophagy important in whole animal physiology and if so, when? Given that Fundc1 itself is eliminated during mitophagy (Liu, L. *et al.*, 2012a), when does mitophagy become rate limiting for mitochondrial function and cell survival? Another interesting question relates to tyrosine18 phosphorylation, which is targeted by Src kinase; given that many Src inhibitors have been developed to prevent growth of cancer cells, it will be interesting to see whether these inhibitors affect Fundc1-dependent mitophagy in cancer cells *in vivo*, especially under hypoxic conditions. This is especially important given that many cancer cell types do not express Parkin, the prime downstream mediator of Pink1-dependent mitophagy, Pink1 still being the best-characterized tag in mammalian cells.

NIX and BNIP3

NIX (also known as BNIP3L) and BNIP3 are bifunctional mitochondrial proteins: they contain a BH3 domain with homology to the BH3-only pro-apoptotic members of the Bcl-2

family proteins and thus can induce cell death (apoptosis, or necrosis following mitochondrial membrane permeabilization). In addition, they can induce autophagy and/or mitophagy through their LIR domains. How their activity is channeled between these two functions is an important question. The discovery and biological functions of this family of proteins has been reviewed by Zhang and Ney (2009) and more recently by Lu *et al.* (2013). In this review we will concentrate on what we know about their roles in mitophagy.

The importance of NIX in mitophagy was first realized when it was shown that reticulocyte to erythrocyte differentiation, during which the cells must rid themselves of mitochondria, does not occur properly in NIX KO mice (Schweers *et al.*, 2007). Mitochondrial elimination was not via properties associated with cell death, or due to activation of autophagy, which was of normal capability in the KO cells, indicating that the mitochondria are removed by mitophagy. However, mitochondrial elimination was not completely suppressed in autophagy-deficient Atg7-KO reticulocytes (Zhang *et al.*, 2009) so there are other mechanisms that can allow erythrocyte maturation, albeit inefficiently. The N-termini of NIX and BNIP3 in human and rodents contain a LIR motif (WVEL) through which they bind differentially to various LC3 family members (Hanna *et al.*, 2012; Novak *et al.*, 2010; Rikka *et al.*, 2011; Schwarten *et al.*, 2009). The importance of the LIR motif was demonstrated by the fact that overexpressed NIX, but not a NIX LIR mutant (NixW35A), restored mitochondrial clearance in NIX KO reticulocytes. BNIP3 is in addition phosphorylated on two serines (17 and 24) flanking the LIR motif and this augments BNIP3's binding to LC3B (and the homologue GATE-16) and enhances the extent of mitophagy. In this case, mitophagy occurred while cytochrome c was still in the mitochondria so it is unlikely to have been the result of mitochondrial membrane permeabilization (Zhu *et al.*, 2013). It should be noted that BNIP3 is also found in the ER so it may not always act as a mitophagy-specific tag (Hanna *et al.*, 2012). An interesting observation was that prosurvival Bcl-xL enhanced BNIP3 binding to LC3B, mitochondrial sequestration, and autophagy, but binding of Bcl-xL to BNIP3 was not via the BH3 domain (which could lead to apoptosis) but rather at the TM domain, through which NIX/BNIP3 form dimers that are necessary for their function (Hanna *et al.*, 2012). Perhaps this binding serves to channel the protein activity towards mitophagy linked to cell survival (Rikka *et al.*, 2011) and at the same time sequester Bcl-xL away from Beclin 1, which further facilitates increased autophagy. Interestingly, ubiquitination of NIX/BNIP3 has not been reported, so p62/SQSTM1 being a factor in NIX/BNIP3 driven mitophagy is still not proven. However, a NIX-dependent recruitment of Parkin was reported in a model of mitophagy induced by drugs that collapse the mitochondrial membrane potential (Ding *et al.*, 2010) and in cardiac myocytes, where overexpressed BNIP3 recruits Parkin to the mitochondria dependent on the activity of the fission-inducing protein dynamin-related protein 1 (Drp1, the mammalian homologue of yeast Dnm1) (Lee *et al.*, 2011). Interestingly, prevention of Drp1 activity or autophagy in BNIP3 overexpressing cells caused cell death, but whether the BH3 domain of BNIP3 was required is not clear. Like Fundc1, both NIX and BNIP3 are highly induced by hypoxia, and overexpression of BNIP3 to levels similar to those found after hypoxia was sufficient to induce autophagy and mitophagy in cells. In summary, NIX is a proven mitochondrial tag with a physiological role whereas questions remain regarding BNIP3: can it operate as an efficient mitochondrial tag on its own and, if not, how does it collaborate with other tags to induce mitophagy? BNIP3 seems to play an important role in cardiac myocytes but how it affects cardiac physiology

remains to be investigated. A BNIP3 (conditional) knockdown transgenic mouse model would clarify the answers to these questions.

Related to the question of the physiological roles of mitophagy in mitochondrial elimination, an ongoing question is how and when paternal mitochondria are deleted from embryos. This topic is reviewed in depth by Luo *et al.* (2013). In *Drosophila* (DeLuca and O'Farrell, 2012), mtDNA is deleted in sperm during spermatogenesis but the involvement of autophagy/mitophagy was not tested. However, Pink1 mutant male flies are sterile and display defects in mitochondrial shape and positioning that persist, showing lack of mitochondrial elimination (Clark *et al.*, 2006). In *C. elegans* embryos, macroautophagy is involved in the degradation of sperm mitochondria and mtDNA by a process that was termed allophagy (Sato and Sato, 2011) and this occurs postfertilization (Al Rawi *et al.*, 2011; Zhou, Q. *et al.*, 2011) but whether mitophagy is specified was not determined. In mice, the sperm tail is prelabeled with ubiquitin before fertilization and co-localizes with p62/SQSTM1 and LC3 proteins after fertilization, suggesting that mitophagy occurs in the oocyte (Al Rawi *et al.*, 2011). However, a direct test of mitochondrial fate after fertilization produced by crossing male and female mice with differentially labeled mitochondria and/or LC3 has shown that most of the sperm cells that have fertilized eggs successfully have already eliminated their mtDNA via autophagy, just leaving vacuolar mitochondria to supply energy for completing fertilization (Luo and Sun, 2013). In rare cases, if a sperm bearing mtDNA is able to fertilize an egg, sperm mitochondria are highly concentrated, and they are then restricted to one blastomere before the 4-cell embryo stage; paternal mtDNA will then be distributed to the placenta or some tissues. Hence, there may not be a mechanism for mitophagic elimination of paternal mitochondria in the zygote.

Pink1 (and Parkin)

Recent studies on Pink1 (PTEN-induced kinase 1, encoded by *PARK6*) and Parkin (encoded by *PARK2*) have yielded the most important insights into mechanism(s) of mitophagy in mammalian cells, aided by finding similar mechanisms in *Drosophila*, where Pink1/Parkin mutants show severe developmental abnormalities allied to mitochondrial dysfunction. A scheme summarizing the main interactions discussed is presented in Figure 3.3.

In humans, Pink1 and Parkin are responsible for most cases of recessive Parkinson's disease. Pink1 is a 581 amino acid protein with a mitochondrial targeting signal at the N-terminus and a serine/threonine kinase domain at the C-terminus facing the cytosol (Zhou, C. *et al.*, 2008). Although a small portion of Pink1 is also present in the cytosol to mediate its protective function (Tieu and Xia, 2014), Pink1 is primarily a mitochondrial protein and, as such, its roles in the mitochondria have been extensively investigated. In healthy mitochondria, Pink1 is imported into inner mitochondrial membrane (IMM) via the translocase of the outer membrane (TOM) and translocase of the inner membrane 23 (TIM23) in a membrane potential dependent manner. Full length Pink1 is then cleaved by mitochondrial processing peptidase (MPP) and presenilin-associated rhomboid-like protease (PARL). Cleaved Pink1 is released into the cytosol to carry out other nonmitochondria-related functions (Dagda *et al.*, 2014) and to be degraded by the proteasome (Yamano and Youle, 2013). Hence Pink1 is maintained at a low level in healthy mitochondria. However,

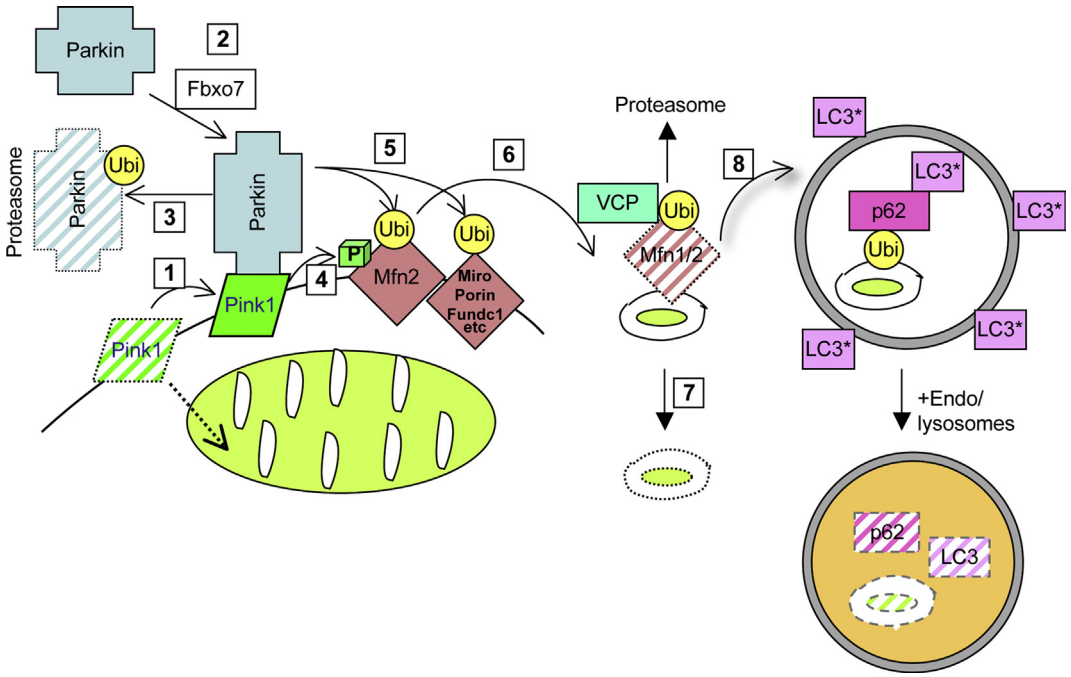

FIGURE 3.3 Suggested mechanism of Pink1 and Parkin mediated mitophagy after mitochondrial damage. 1. Pink1 stabilization; 2. Recruitment of Parkin from the cytoplasm to the mitochondria; 3. Destruction of Parkin via the proteasome in balance with steps 1 and 4; 4. Phosphorylation of IMM proteins by Pink1; 5. Ubiquitination of IMM proteins. 6. Proteasomal destruction of mitofusins via ubiquitination and fission; 7. Segregation of some mitochondrial proteins away from the phagosome; 8. Engulfment of the mitochondrial body, fusion with endo/lysosomal compartments and degradation.

in mitochondria with disrupted membrane potential, Pink1 is not imported to the IMM but rather it is directed to the outer mitochondrial membrane (OMM), resulting in a rapid accumulation of full length Pink1 there. At OMM, Pink1 recruits Parkin from the cytosol to mitochondria. Structurally, Parkin has a RING domain at the C-terminus and an ubiquitin-like domain at the N-terminus. As a multifunctional E3 ubiquitin ligase, Parkin is capable of tagging ubiquitin to proteins at K48 for proteasomal degradation or at K63 for other biological functions. Parkin has been well reported to function downstream of Pink1 in both *Drosophila* and mammalian cells.

In membrane potential depolarized mitochondria, cytosolic Parkin is recruited to the mitochondria and binds to Pink1. This recruitment depends on the kinase activities of Pink1; however, it is not yet clear how Pink1 activates and selectively directs Parkin to mitochondria, though recent evidence points to Pink1-mediated Parkin self-association required for its E3 ubiquitin ligase activity (Lazarou *et al.*, 2013) and the protein F-box domain-containing protein Fbxo7 (encoded by PARK15) as a possible chaperone, whose expression rescued Parkin KO cells in *Drosophila* (Burchell *et al.*, 2013). Although Pink1 and Parkin bind

to each other, it is still a topic of debate whether these two proteins can phosphorylate and ubiquitinate, respectively, each other.

Once recruited, Parkin initiates the process of mitophagy to eliminate damaged mitochondria. The potential mechanisms by which Pink1/Parkin promotes mitophagy have been extensively reviewed (Ashrafi and Schwarz, 2013; de Vries and Przedborski, 2013; Narendra *et al.*, 2012; Van Laar and Berman, 2013) and thus will not be discussed in detail here. In brief, three nonmutually exclusive mechanisms have been proposed after Parkin has been recruited to mitochondria: First, Parkin conjugates ubiquitin to outer mitochondrial membrane (OMM) proteins, including mitofusins 1 and 2 (mfn1/2), which promote mitochondrial fusion. The subsequent degradation of these mitochondrial fusion proteins, which may be facilitated by their VCP/p97dependent targeting to the proteasome (Kim *et al.*, 2013), leads to mitochondrial fission. Thus, degradation of mitofusins may serve to segregate and remove dysfunctional mitochondria through enhanced mitophagy. This proposed mechanism fits well with the mitochondrial fission model as a mitochondrial quality control mechanism in cells (Twig *et al.*, 2008) that is also relevant to yeast, as discussed above. The translocase of the outer mitochondrial membrane (TOMM) import machinery has also been implicated in Pink1/Parkin-dependent mitophagy; blocking import via the TOMM triggers Pink1-dependent recruitment of Parkin, a decrease of TOMM components is required for Pink1/Parkin-mediated mitophagy, while overexpressed TOMM components reduce Pink1/Parkin-dependent clearance of mitochondria induced by mitochondrial depolarization (Bertolin *et al.*, 2013). Indeed, Tomm70a, a key component of TOMM, is heavily ubiquitinated by Parkin, especially notable when proteasome and lysosome activities are inhibited (Sarraf *et al.*, 2013).

Second, in concerted efforts, Pink1 and Parkin arrest the anterograde transport of dysfunctional mitochondria by degrading Miro, which links mitochondria to microtubules by binding to kinesins. The loss of this OMM protein results in the detachment of mitochondria from the microtubules and hence trafficking (Kane and Youle, 2011; Liu, S. *et al.*, 2012b; Sarraf *et al.*, 2013; Wang, X. *et al.*, 2011b). Third, increased mitochondrial ubiquitination directly leads to recruitment of various autophagic proteins, in particular p62/SQSTM1 (Johansen and Lamark, 2011; Kirkin *et al.*, 2009). A recent in-depth analysis of the Parkin-dependent ubiquitinome in response to mitochondrial depolarization has revealed several new proteins that are ubiquitinated by Parkin – not all of which are mitochondrial (Sarraf *et al.*, 2013). Sorting out which are specifically required for mitophagy, especially *in vivo*, will be important for a deeper understanding of the mechanisms of mitophagy in mammalian cells.

In *Drosophila*, a Pink1 loss of function mutant results in male sterility, muscle degeneration, defects in mitochondrial morphology and increased sensitivity to multiple stresses, which is partially reversed by expressing human Pink1. Pink1 localizes to mitochondria, and mitochondrial cristae are fragmented in Pink1 mutants. Loss of Parkin phenocopies, and loss of function of Pink1, and its overexpression rescues the male sterility and mitochondrial morphology defects of Pink1 mutants, placing Parkin downstream of Pink1 (Clark *et al.*, 2006). Recent work has further shown that the *Drosophila* homologue of p62/SQSTM1, *ref(2)P*, is necessary for the rescue of the Pink1 mutant phenotype by Parkin (de Castro *et al.*, 2013) and requires both its ubiquitin binding domain (UBA) and its multimerization domain (PB1). In the absence of *ref(2)P*, sperm mitochondria were abnormal, mtDNA mutations arose, motor function was decreased during aging and flies had a shortened life span.

Most importantly, autophagy was necessary for the rescue of the Parkin-dependent Pink1 mutant fly phenotype.

In support of the beneficial effects of Pink1/Parkin mediated mitophagy in flies, Parkin overexpression decreased protein aggregation via its ubiquitin ligase activity, increased life span, and decreased the deleterious rise in mitofusins (which presumably prevents fission required for mitophagy) that occurs during aging (Rana *et al.*, 2013). Furthermore, *Drosophila* E2 ubiquitin ligase dRad6 cooperates with Parkin to ubiquitinate mitochondrial proteins and facilitate the clearance of dysfunctional mitochondria. Thus, dRad6 mutants have defective synaptic function due to mitochondrial failure. Interestingly, mouse mRad6a (Ube2a) knockout and patient-derived hRad6a (Ube2a) mutant cells also contain defective mitochondria. The *Drosophila* orthologue of insulin-like growth factor-binding protein 7 (IGFBP7) is a novel regulator of mitophagy; deletion of NDUFS1/ND75, a component of mitochondrial complex I, which induces "mild muscle mitochondrial distress" via increased ROS, prolongs fly lifespan. The ROS activates a mitochondrial unfolded protein response (UPR(mt)), thereby helping to restore mitochondrial and muscle function. In addition, it induces systemic production of IGFBP7, which binds to insulin, thereby increasing fly life span. The mitochondria recruited autophagosomes, and autophagy was necessary for the beneficial effect of IGFBP1, but it is not clear whether Pink1/Parkin mediates this effect. The authors propose that the increase in autophagy is due to an increased number of lysosomes rather than increased autophagy *per se* (Owusu-Ansah *et al.*, 2013).

In recent years the topic of mitophagy as a mitochondrial quality control mechanism mediated by Pink1 and Parkin in mammalian systems has been intensively investigated mainly in the field of neurodegenerative disorders. However, to date, many questions and issues remain to be addressed. For example, (1) what is the physiological relevance of this pathway in the mammalian brain? Most of the studies that characterize the effects of Pink1 and Parkin on mitophagy have been carried out in nonneuronal or immortalized cells such as HeLa, MEF, and SH-SY5Y cells. In induced pluripotent stem (iPS) cell-derived neurons (Rakovic *et al.*, 2013) or primary neurons (Van Laar *et al.*, 2011), overexpressing Parkin does not lead to measurable mitophagy. An *in vivo* model with mitochondrial dysfunction in dopaminergic neurons due to loss of the key mitochondrial transcription factor TFAM (MitoPark mice) also fails to detect Parkin-mediated mitophagy in the brain (Sterky *et al.*, 2011). MitoPark mice (where lack of TFAM in dopaminergic neurons leads to mitochondrial DNA depletion and neurodegeneration) crossed with Parkin-knockout mice did not exhibit more accumulation of damaged mitochondria and neurodegeneration. Even when Parkin was overexpressed in these MitoPark mice, it was not recruited to mitochondria. Together, these data indicate that Parkin does not play a role in mitophagy in the brain *in vivo*, at least in this setting. (2) To induce mitochondrial dysfunction, protonophores such as CCCP are frequently used to collapse membrane potential. However, this is most likely physiologically irrelevant. Furthermore, CCCP has recently been reported to interfere with the lysosomal function and autophagosomal degradation (Padman *et al.*, 2013). (3) The physiological relevance of Pink1 or Parkin-dependent mitophagy is also lacking due to the routine use of high transfection of these proteins. At endogenous levels, Parkin fails to mediate mitophagy in human primary fibroblasts and iPS-derived neurons (Rakovic *et al.*, 2013). (4) Another issue to consider is the proposal that by ubiquitinating mitofusins Parkin promotes fission, therefore facilitating the removal of damaged mitochondria through mitophagy. It is still

controversial whether Parkin promotes fission or fusion in mammalian cells. Parkin has been reported by various laboratories to have mitochondrial pro-fusion effects (Cui *et al.*, 2010; Dagda *et al.*, 2009; Exner *et al.*, 2007; Lutz *et al.*, 2009). To add complexity to the role of Parkin on mitochondrial dynamics, Parkin was also reported to downregulate the levels of the mitochondrial fission proteins Drp1 (Wang, H. *et al.*, 2011a) and Fis1 (Chan *et al.*, 2011; Cui *et al.*, 2010), while a more recent study of the depolarization-induced Parkin ubiquitinome finds low Fis1 modification (Sarraf *et al.*, 2013). Interestingly, the latter study found a large extent of modification of subunits of the catalytic and regulatory particles of the proteasome, agreeing with Chan *et al.* (2011) that Parkin's roles are not confined to mitophagy. Moreover, Parkin has been recently implicated in ubiquitin-mediated autophagy of *M. tuberculosis* and in quelling bacterial infection in both mice and *Drosophila* (Manzanillo *et al.*, 2013). Further supporting the notion that loss of mitofusin alone is not sufficient to induce mitophagy, Parkin-mediated mitophagy is still blocked by inhibition of the proteasome, whereas the ubiquitination of several OMM proteins accumulates upon inhibition of the proteasome, suggesting other mechanisms are also involved (Chan *et al.*, 2011; Sarraf *et al.*, 2013). In summary, although extensively characterized in cultured cells, the physiological relevance and significance of Pink1 and Parkin-dependent mitophagy remain to be established, especially *in vivo*.

GENERAL CONCLUSIONS

Remarkable advances in our understanding of mitophagy have occurred in the last five years (Tolkovsky, 2009). As in autophagy, studies on yeast have clarified the fundamental principles of mitophagy that serve as a guide for studies of mitophagy in metazoans. What is needed for mitophagy to occur is (1) a mitochondrial tag that is marked so that the damaged region of the mitochondrion is specified, (2) recruitment of proteins that bridge to and/or comprise components of the autophagosome, and (3) fragmentation of the mitochondria, most likely for selection of those components that need to be delivered to the lysosome for degradation via mitophagy. In metozoans, it seems that there are several proteins that can serve as tags, probably in order to meet various conditions that either reduce the need for mitochondria or get rid of faulty mitochondria. When these conditions are not met, mitochondria will accumulate inappropriately, and lead to cell dysfunction and/or death, albeit in some cases the boundaries between mitophagy and autophagy are blurred, and mitophagy *per se* can lead to cell death. A current challenge is to clarify, in mammals when mitophagy is required specifically, and when mitochondrial degradation is part of the general autophagic process. This pertains especially to the brain in neurodegenerative disorders. Another crucial point is to determine when the commitment point is reached beyond which mitochondrial biogenesis cannot repair degradation. Clearly, it is important to understand the balance between mitophagy and biogenesis in each organ, as this will determine whether the organism will live or die.

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CHAPTER

4

Quality Control in Mitochondria

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OUTLINE

Abstract

Mitochondria are essential components of the eukaryotic cell, containing the machinery for efficient ATP production by oxidative phosphorylation and other metabolic processes. However, a side effect of these reactions is the generation of reactive oxygen species (ROS), which are deleterious to the integrity and functioning of mitochondria and to the cells which harbor them. Fortunately, cells possess a number of mechanisms to counter ROS damage in mitochondria, including repair, bypass reactions, and the degradation of damaged proteins and organelles. This final process occurs by utilizing the autophagic machinery to target damaged mitochondria for vacuolar degradation, and is termed mitophagy. This review discusses different mechanisms of mitochondrial quality control in yeast cells, with an emphasis on mitophagy.

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MECHANISMS OF MITOCHONDRIAL QUALITY CONTROL

Reactive oxygen species (ROS) are generated from respiratory metabolism (Costa *et al.*, 1997) but also in response to external stimuli (Jamieson, 1998; Perrone *et al.*, 2008). ROS inflict damage upon mitochondrial DNA (Yakes and Van Houten, 1997), proteins (Cabiscol *et al.*, 2000) and lipids (Bilinski *et al.*, 1989). In addition to ATP generation by oxidative phosphorylation, mitochondria play a crucial role in amino acid, haem, and isoprenoid biosynthesis. In order to preserve mitochondrial function in the face of self-inflicted damage, cells have evolved a variety of techniques to repair damage if possible or, failing that, to degrade entire organelles. Upon mitochondrial dysfunction, cells can express alternative enzymes for the production of α-ketoglutarate thus bypassing the requirement for the TCA cycle for amino-acid synthesis. Proteolytic degradation of damaged proteins by resident mitochondrial proteases (Bender *et al.*, 2011; Graef *et al.*, 2007; Kaser *et al.*, 2003) and a retro-translocation system for misfolded mitochondrial outer membrane proteins, similar to ERAD (Heo *et al.*, 2010), have also been reported. Damaged mitochondria have also been shown to be selectively degraded through autophagy, a process that is termed mitophagy (Lemasters *et al.*, 1998).

Autophagy in *S. cerevisiae*

Autophagy is a catabolic process that occurs in all eukaryotic cells. Autophagy can be nonselective or selective. Nonselective autophagy normally occurs in response to nitrogen starvation, and involves the sequestration of bulk cytoplasm into double-membrane vesicles (autophagosomes) that fuse with the lysosome/vacuole, where the contents of the autophagosome are degraded (Xie and Klionsky, 2007). Selective autophagy can involve the degradation of specific unnecessary or dysfunctional cellular components, molecules as well as organelles. In this category we can include the targeting of specific proteins (such as Ape1, Lap3 or Ald6 [Kageyama *et al.*, 2009; Nice *et al.*, 2002; Onodera and Ohsumi, 2004]) or organelles as cargoes for autophagy. Examples of organelles that undergo selective autophagy are mitochondria (mitophagy) (Lemasters, 2005), peroxisomes (pexophagy) (Hutchins *et al.*, 1999), endoplasmic reticulum (reticulophagy/ERphagy) (Bernales *et al.*, 2007), and nucleus (late nucleophagy); and also piecemeal microautophagy of the nucleus (Mijaljica *et al.*, 2012b) and ribosomes (ribophagy) (Kraft *et al.*, 2008). A general model for selective autophagy involves a ligand located on the target interacting with a specific receptor which in turn binds a scaffold that links the targeted organelle to the autophagy machinery (Mijaljica *et al.*, 2012a). Using mitophagy in *S. cerevisiae* as an example, Atg32 functions as the specific receptor which binds to the Atg11 scaffold (Okamoto *et al.*, 2009). Atg11 is required for recruitment of the mitochondria in question to atg8-PE (Kondo-Okamoto *et al.*, 2012; Okamoto *et al.*, 2009), which lines the initial sequestering compartment.

An Overview of Mitophagy in *S. cerevisiae*

The first report of mitochondria within structures that were later identified as autophagolysosomes in mammalian cells was in 1957 (Clark, 1957). Five years later autophagy was

discovered; it was demonstrated that the number of lysosomes in rat hepatocytes following glucagon treatment is elevated, and that these lysosomes often contain mitochondria (Ashford and Porter, 1962). A study of ultrastructural changes in the intersegmental muscles during programmed cell death in the metamorphosing muscles of the silk moth led to the proposition that mitochondria undergo selective removal during metamorphosis. Whereas other organelles appeared to be normal, mitochondria underwent both functional and morphological changes leading to their engulfment by autophagosomes (Beaulaton and Lockshin, 1977). In 1980, the induction of autophagy of damaged organelles of the rabbit heart, especially of mitochondria, was shown to be connected with subcellular damage caused by hypoxia and the recovery from hypoxia (Decker and Wildenthal, 1980). A quantitative ultrastructural investigation by Heynen and Verwilgen of normal rat erythroblast and reticulocytes showed complete elimination of mitochondria (and a reduction in the levels of other organelles of the secretory pathway) during their maturation. It was suggested that mitochondria are recycled through an autophagy-like process (Heynen and Verwilghen, 1982). The mechanism of this regulated clearance of mitochondria was not well understood until the finding that the Bcl-2-related protein NIX is required for programmed elimination of mitochondria during reticulocyte maturation (Sandoval *et al*., 2008; Schweers *et al*., 2007), with NIX functioning as a mitochondrial receptor for the autophagy machinery (Novak *et al*., 2010) (see below).

Autophagy in yeast has been investigated since 1992, when Takeshige *et al*. (1992) found autophagic vesicles inside vacuoles following nitrogen starvation under fermentative conditions. Subsequent genetic screens identified a host of genes (now termed ATG but initially possessing a plethora of confusing nomenclature (Klionsky *et al*., 2003) involved in autophagy (Barth and Thumm, 2001; Harding *et al*., 1995, 1996; Thumm *et al*., 1994; Tsukada and Ohsumi, 1993).

Three conditions are widely used in *S. cerevisiae* to induce mitophagy. Mitophagy was first observed following a switch from rich growth media with lactate as the carbon source (which is respired) to synthetic media with glucose but lacking nitrogen (herein abbreviated as YPL > SD-N), thus simultaneously repressing respiration but imposing starvation to induce autophagy (Kanki and Klionsky, 2009; Kanki *et al*., 2009a; Kissova *et al*., 2004; Okamoto *et al*., 2009; Priault *et al*., 2005). Rapamycin (an inhibitor of TORC1) treatment also elicits mitophagy in respiring cells (Kissova *et al*., 2004), but on a slower timescale and with less potency than YPL > SD-N (Mao *et al*., 2013). Mitophagy also occurs following extended stationary phase incubation in respiratory conditions (Tal *et al*., 2007) and since this effect can be uncoupled from starvation, it is thought to be the result of quality control events. Genetic investigations using the single knockout collection identified genes that are involved in stationary-phase mitophagy. Most of these are factors that are also required for general starvation-mediated autophagy but two mitophagy specific proteins (Atg32, Atg33) (Kanki *et al*., 2009a; Okamoto *et al*., 2009) have also been identified.

Additional conditions reported to induce mitophagy include anaerobic incubation in YPD medium of *fmc1Δ* cells at 37°C (Priault *et al*., 2005), which is nonpermissive for the correct assembly of the F_1F_0 ATPase (Lefebvre-Legendre *et al*., 2001). Although these conditions resulted in the apparent degradation of the mitochondrial proteins Por1 and Atp1, mitophagy *per se* was not visualized in this study. Treatment of rho° cells with bongkreic acid (in YPD medium) weakly induces autophagy (Priault *et al*., 2005). A similar technique

to induce mitophagy involves a shut-off of *MDM38* expression (Nowikovsky *et al.*, 2007). Mdm38 is a K^+/H^+ antiporter. *mdm38Δ* cells show functional defects of mitochondria, reduced growth of cells on nonfermentable substrate, a nearly complete loss of mitochondrial $K^+(Na^+)/H^+$ exchange activity, high K^+ matrix content, low membrane potential ($\Delta\psi$), and dramatically increased volume (Froschauer *et al.*, 2005; Nowikovsky *et al.*, 2004). Under these conditions, mitophagy was observed in galactose containing media (Nowikovsky *et al.*, 2007). Both of these methods have the common motif that they introduce a genetic lesion causing a defect mitochondrial function leading to mitophagy.

Additional Mitochondrial Quality Control Mechanisms

In addition to elimination of defective mitochondria by mitophagy, *S. cerevisiae* possesses at least two other mechanisms to degrade damaged mitochondrial proteins. Mitochondria possess internal proteases that are involved in maturation of proteins required for respiration (Suzuki *et al.*, 1994; Thorsness *et al.*, 1993) and in the degradation of damaged proteins. Yme1 is an AAA+ ATPase at the mitochondrial inner membrane, with a metallopeptidase domain exposed to the intermembrane space (Graef *et al.*, 2007). It degrades misfolded, damaged, or misassembled proteins as part of mitochondrial protein quality control (Baker *et al.*, 2012; Graef *et al.*, 2007). The function of Yme1 in mitophagy is controversial. Yme1 has been proposed to be required for mitophagy induced maturation of Atg32 (Wang *et al.*, 2013), but *yme1Δ* cells show an increased mitophagy rate under respiratory conditions (Campbell and Thorness, 1998; Welter *et al.*, 2013).

Oma1 is a conserved peptidase that degrades misfolded inner-membrane proteins (Kaser *et al.*, 2003). Whereas neither deletion of *OMA1* nor *YME1* nor *VMS1* results in an absence of growth on glycerol, the double mutants *oma1Δyme1Δ* and *oma1Δvms1Δ* are incapable of respiratory growth (Heo *et al.*, 2010).

ROS production also stimulates expression of the Pim1 protease, which degrades many ROS damaged soluble proteins of the matrix (Bender *et al.*, 2010). Recognition of damaged proteins by Pim1 is partially dependent upon Hsp78 (Leidhold *et al.*, 2006). Sixteen mitochondrial proteins have been identified as being aggregation prone following heat stress (42°C), which is also a source of ROS. Aggregation disrupts several key metabolic processes, including the TCA cycle. Pim1 is important in the clearance of these aggregates (Bender *et al.*, 2011). The relationship of Oma1 and Pim1 to mitophagy has not been determined.

Mutation of Cdc48 (S56G), a chaperone involved in the unfolded protein response of the endoplasmic reticulum (Rabinovich *et al.*, 2002), causes respiratory deficiency and ROS production (Braun *et al.*, 2006). Cdc48 interacts with different cofactor proteins that target Cdc48 activity to distinct cellular sites and also mediate ubiquitin and proteasome binding (Jentsch and Rumpf, 2007). $Cdc48^{S565G}$ has impaired interaction with Vms1, with both $Cdc48^{S565G}$ and *vms1Δ* cells exhibiting accumulation of Fzo1 (Heo *et al.*, 2010). Vms1 is required for protein retro-translocation from mitochondria to the cytosol (Heo *et al.*, 2010), and is recruited to oxidatively damaged mitochondria (Heo *et al.*, 2013). *vms1Δ* cells also exhibit increased stationary-phase mitophagy (Heo *et al.*, 2010), demonstrating how absence of one quality control mechanism upregulates an alternative system of maintaining healthy mitochondria.

Whereas damaged proteins are removed from mitochondria by pathways leading to their degradation (whether within the mitochondria or in the cytosol), oxidized lipids can be repaired by glutathione peroxidases (Gpx1, Gpx2, and Gpx3). Phospholipid hydroperoxide glutathione peroxidase activity is reduced in single mutants of *gpx1Δ*, *gpx2Δ*, or *gpx3Δ* and is almost completely abolished in the triple deletion mutant (Avery and Avery, 2001). Gpx1 expression is induced by glucose starvation (Inoue *et al*., 1999) and more strongly by oleic acid in an Msn2/Msn4 dependent manner, and is extrinsic to the mitochondrial outer membrane and also shows localization to the matrix of peroxisomes. *gpx1Δ* cells grow poorly when oleic acid is the sole carbon source in the external medium. Gpx1 uses glutathione or thioredoxin as a reducing agent (Ohdate and Inoue, 2012). Gpx2 is localized to both the cytosol and mitochondria (Ukai *et al*., 2011), and its expression is induced by oxidative stress via signaling to the Yap1 transcription factor (Inoue *et al*., 1999). Gpx2 is localized to the outer leaflet of the outer mitochondrial membrane, and to the matrix side of the inner membrane. Gpx2 prefers to use thioredoxin as its reducing agent. Although *GPX1* or *GPX2* deletions do not show sensitivity to peroxides and are functionally redundant during vegetative growth (Inoue *et al*., 1999), *gpx2Δ* cells exhibit defective sporulation (Ukai *et al*., 2011).

GPX3 expression is constitutive (Inoue *et al*., 1999) and the Gpx3 protein is localized to both the peroxisomal matrix (Ohdate and Inoue, 2012) and the mitochondrial intermembrane space (Vogtle *et al*., 2012). In addition to its phospholipid hydroperoxide glutathione peroxidase activity (Avery and Avery, 2001; Delaunay *et al*., 2002; Inoue *et al*., 1999), Gpx3 activates the Yap1 transcription factor (Avery *et al*., 2004; Delaunay *et al*., 2002) by catalyzing the formation of an intramolecular di-sulfide bridge within Yap1, causing its nuclear retention (Delaunay *et al*., 2002). Yap1 induces the transcription of many genes involved in oxidative stress protection, such as the catalases *CTA1* and *CTT1* (Ouyang *et al*., 2011) and *GPX2* (Inoue *et al*., 1999). Whether this lipid repair and antioxidant stress response system relates to mitophagy and other mitochondrial quality control mechanisms has not been determined at the time of writing.

CAUSES OF MITOPHAGY

The observation that mitophagy is induced as a consequence of prolonged respiration (Tal *et al*., 2007) suggests that ROS may be involved in the induction of mitophagy. Alternatively, yeast cells growing in nitrogen-rich medium containing a nonfermentable carbon source transferred to a minimal medium lacking nitrogen but containing glucose (which immediately inhibits respiration; for brevity we will refer to this procedure as YPL > SD-N) quickly induce mitophagy, thus potentially lowering ROS production. In contrast, damaged mitochondria in mitophagy-deficient yeast suffer increased ROS production in a vicious feed-forward cycle eventually resulting in deletion of mitochondrial DNA (Kurihara *et al*., 2012). The antioxidant N-Acetyl-L-cysteine (NAC) or cysteine itself inhibits mitophagy induced by YPL > SD-N by elevating the levels of reduced glutathione (GSH) (Deffieu *et al*., 2009). L-cysteine is the precursor of glutathione but free L-cysteine is present in only low quantities in the cell, so raising its levels has a direct impact on glutathione production. This effect was specific to cysteine and does not occur when other amino acids are supplemented and so is not due to alleviation of nitrogen starvation. Lowering the intracellular

pool of GSH using ethacrynic acid promotes mitophagy under both YPL > SD-N and stationary phase conditions (Deffieu *et al.*, 2009). Levels of Atg32, which correlate with the rate of mitophagy, are lowered by NAC (Okamoto *et al.*, 2009). Addition of rapamycin to cells grown under respiratory conditions induces lipid oxidation and mitophagy. Rapamycin, but not YPL > SD-N induced mitophagy is inhibited by resveratrol (Kissova *et al.*, 2006). Resveratrol is a mild oxidant that induces expression of Yap1 dependent genes, thus provoking antioxidant responses and protection (Escote *et al.*, 2012).

It is important to note that while increasing or decreasing ROS promotes or inhibits mitophagy respectively, the relevant targets of ROS for the promotion of mitophagy and signaling mechanisms to link ROS damage to induction of mitophagy have not been identified.

YEAST PROTEINS INVOLVED IN MITOPHAGY

ATG Proteins

At least 33 genes whose products are involved in autophagy (ATG genes) have been identified to date, with roles in the cytosol to vacuole transport (Cvt) pathway, nonspecific micro and macro autophagy, and also for selective micro and macro autophagy of organelles. About half of the Atg proteins comprise the common components required for the assembly of autophagosomes, with other Atg proteins providing adaptors for specific organelles. Atg1, 5–9, 12 and 13 are required for bulk-autophagy and mitophagy (Kissova *et al.*, 2004, 2007; Tal *et al.*, 2007; Zhang *et al.*, 2007). Atg11 was first identified as an adaptor for pexophagy and the transport of Ape1 to the vacuole by the Cvt pathway (Kim *et al.*, 2001) and has since been found to be required for mitophagy under both YPL > SD-N and stationary phase conditions (Kanki and Klionsky, 2008; Okamoto *et al.*, 2009) but not for bulk autophagy (Kim *et al.*, 2001).

ATG32 was identified by two yeast genetic screens to be indispensable for mitophagy but not for nitrogen-starvation-induced macroautophagy (Kanki *et al.*, 2009a,b; Okamoto *et al.*, 2009). Atg32 is an integral membrane protein localized to the mitochondrial outer membrane. *ATG32* expression is induced by respiratory growth and lowered by the antioxidant NAC (Okamoto *et al.*, 2009). Induction of mitophagy correlates with phosphorylation of S114 and S119 (Aoki *et al.*, 2011) on Atg32, permitting interaction of the N-terminus of Atg32 (residues 51–150) with the C-terminus of Atg11 (the cytosolic scaffold for selective autophagy) (Okamoto *et al.*, 2009). Atg32 can also interact with Atg8 via a conserved W/YxxI(/L/V) motif in its cytosolic domain (Okamoto *et al.*, 2009). Thus, Atg32 mediates mitophagy via both direct and indirect interaction with Atg8. Of these two phosphorylated serines, phosphorylation of S114 is of greater importance for this interaction. Atg11 then recruits the mitochondrion to the sequestering autophagic membrane (Aoki *et al.*, 2011). Under YPL > SD-N conditions, CK2 is required for phosphorylating Atg32 at both of these sites within 1 hour of nitrogen starvation. Hog1, but not Slt2, is upstream of CK2 in this pathway, though direct phosphorylation of CK2 by Hog1 has not been observed. CK2 is not required for bulk autophagy or pexophagy (Kanki *et al.*, 2013). Since both CK2 and Hog1 regulate multiple processes that do not induce mitophagy, it remains to be determined how

this signaling pathway induces mitophagy under YPL > SD-N conditions. Interestingly, an as-yet unidentified residue in Atg32 is rapidly **de**phosphorylated upon induction of mitophagy (Aoki *et al.*, 2011; Kanki *et al.*, 2013).

Atg33 was identified in one screen of single-knockout mutants as being involved in mitophagy (Kanki *et al.*, 2009a). Atg33 is localized to the outer face of the outer mitochondrial membrane and is required for mitophagy and not for other processes such as pexophagy (Kanki *et al.*, 2009a). Although deletion of *ATG33* almost completely prevents stationary phase induced mitophagy, nitrogen starvation coupled with carbon source switch induced mitophagy is only reduced by about 50% (Kanki *et al.*, 2009a; Welter *et al.*, 2013). This suggests that these different conditions may induce mitophagy through different mechanisms (Kanki *et al.*, 2009a). As well as mitophagy specific adaptors, some proteins required for bulk autophagy (e.g. Atg29) or the Cvt pathway (e.g. Atg19) are not required for mitophagy (Okamoto *et al.*, 2009).

MAP Kinases

Mao *et al.* (2011) identified two MAP kinase pathways that are involved in both YPL > SD-N and stationary phase mitophagy. Both Slt2 and Hog1 are required for mitophagy, with Slt2 being activated earlier than Hog1 (Mao *et al.*, 2011). Slt2 is required for recruitment of mitochondria to the pre-autophagosomal structure. The complete cell-wall integrity pathway comprising Pkc1, Bck1, Mkk1/2 and Slt2 (Levin, 2005) has been reported to be required for mitophagy, with the upstream sensor being Wsc1. The upstream input for the Hog pathway is Sln1 (Mao *et al.*, 2011). This is intriguing since both Wsc1 and Sln1 are cell-surface proteins and have not been found in or associated with mitochondria. Both Hog1 and Slt2 remain in the cytoplasm during mitophagy, suggesting that they activate currently unidentified cytoplasmic targets (Mao *et al.*, 2011). Hog1 and its upstream kinase Pbs2 have recently been demonstrated to regulate the phosphorylation of Atg32 by CK2 (Aoki *et al.*, 2011; Kanki *et al.*, 2013). Whereas the Hog pathway is not involved in bulk autophagy, the Slt2 pathway is also required for pexophagy (Mao *et al.*, 2011).

In contrast to the rather incomplete information about signaling pathways involved in mitophagy in yeast (where some kinases and phosphatases have been found but their regulation and targets is unclear), at least one mitophagy-promoting signaling pathway has been identified in mammalian cells: The mitochondrial outer membrane protein FUNDC1 is required for hypoxia-induced mitophagy through the interaction of its YxxL motif with Atg8/LC3 (Liu *et al.*, 2012). The tyrosine in this motif is phosphorylated under ambient conditions by Src (Liu *et al.*, 2012) which also phosphorylates several other mitochondrial proteins (Livigni *et al.*, 2006; Ogura *et al.*, 2012). Under hypoxic conditions, Src is inactivated, FUNDC1 concomitantly dephosphorylated, and its interaction with Atg8/LC3 enhanced (Liu *et al.*, 2012).

Phosphatases

Aup1 is a protein phosphatase with high similarity to murine PPC2m/PP1K (Lu *et al.*, 2007) and is conserved among eukaryotes. Aup1, as well as its mammalian homologues, are

either predicted or known to localize to mitochondria in their respective cells. Aup1 was reported to localize to the intermembrane space of mitochondria, and to be associated with the inner membrane (Tal *et al.*, 2007). Aup1 was identified as being involved in autophagic processes in a screen for phosphatase homologues that genetically interact with the autophagy kinase Atg1 (Ruan *et al.*, 2007; Tal *et al.*, 2007). Aup1Δ cells show defects in both stationary phase (SL) mitophagy (Journo *et al.*, 2009; Tal *et al.*, 2007) and rapamycin-induced mitophagy (Gonzalez *et al.*, 2013). One target of Aup1 has been reported in *S. cerevisiae*: Pda1 (pyruvate dehydrogenase), which converts pyruvate into the acetyl moiety of acetyl-CoA (Gey *et al.*, 2008), but this is not involved in mitophagy (Gonzalez *et al.*, 2013).

Aup1 is required for the retrograde (RTG) signaling pathway (Journo *et al.*, 2009), specifically for signaling to Rtg3 during growth on lactate and for induction of genes such as CIT2 for Krebs cycle metabolism when mitochondria are stressed (Liu and Butow, 1999). The RTG pathway is in turn required for mitophagy (Journo *et al.*, 2009). However, the activator and target(s) of Aup1 during mitophagy have not been identified. Although deletion of either *AUP1* or *RTG2* causes hyperaccumulation of glycogen (Wilson *et al.*, 2002) a mechanistic connection between mitophagy or the RTG pathway and glycogen metabolism has not been determined.

The RTG (Retrograde) Signaling Pathway and Mitophagy

The RTG pathway is activated under conditions of mitochondrial stress and results in the expression of genes whose products enable the production of α-ketoglutarate in the absence of functioning mitochondria. Rtg2 has been reported to relieve the inhibition of Rgt1/Rgt3 by Mks1, a function that is possibly mediated through dephosphorylation of Rtg3 (Liu *et al.*, 2003). This causes the translocation of the heterodimeric Rtg1/Rtg3 transcription factor to the nucleus, where it activates transcription of a set of genes that reprogram metabolism to bypass mitochondria (reviewed in Liu and Butow, 2006). Rtg3 is required for mitophagy (Journo *et al.*, 2009), suggesting linkage between these pathways. However, the downstream effector of the RTG pathway which is required for mitophagy is currently unknown.

Uth1

Uth1 was originally identified in a screen of oxidative-stress response genes, with mutants exhibiting increased survival following starvation and a longer chronological lifespan. However, deletion of *UTH1* retards growth in rho° cells (Bandara *et al.*, 1998). Uth1 is a SUN domain protein and has been reported to localize to both mitochondria and the cell wall (Velours *et al.*, 2002). Uth1 has been shown to be a negative regulator of cell wall construction (Ritch *et al.*, 2010). Uth1 was proposed to be an adaptor (similar in function to Atg32, which at the time had not been discovered) linking mitochondria to the autophagic machinery (Kissova *et al.*, 2004); however, this is controversial due to Uth1's localization to the **inner** mitochondrial membrane (Welter *et al.*, 2013). Whereas the studies identifying Uth1 as a factor required for mitophagy were conducted using the W303 genetic background (Kissova *et al.*, 2004), in the BY background Uth1 is dispensable for mitophagy (Welter *et al.*, 2013). The differences may be accountable due to the report that W303 carries the y*bp1-1* mutation which increases the sensitivity of cells to oxidative stress by decreasing Yap1 activation (Okazaki *et al.*, 2005).

Functional Homologues of Yeast Mitophagy Proteins in Mammals

Although Atg11 and Atg32 do not have homologues in metazoans (Kanki *et al.*, 2010), functional equivalents of these proteins do occur in mammals. The ubiquitin-binding adaptor p62 (SQSTM1) seems to have a role similar to Atg11, as it accumulates on damaged mitochondria. The role of p62 in mitophagy is controversial, however (Geisler *et al.*, 2010; Narendra *et al.*, 2010b). Another protein, Nix, could potentially fulfill the functions of both Atg32 and Atg11 as it resides on mitochondria and binds directly to Atg8/LC3. Nix is induced during reticulocyte development and is required for the subsequent clearance of mitochondria in erythrocytes (Novak *et al.*, 2010; Sandoval *et al.*, 2008; Schweers *et al.*, 2007). NIX has a WxxL motif near its N-terminus, mutation of which reduces mitochondrial clearance (Novak *et al.*, 2010). However, the major role of Nix seems to be in promoting apoptosis: Transient expression of Nix induces and promotes cell death in several cell lines (Chen *et al.*, 1999; Farooq *et al.*, 2001; Imazu *et al.*, 1999; Yussman *et al.*, 2002). Knockout of Nix promotes tumorigenesis, whereas its transient overexpression inhibits colony formation in several cancer cell lines (Fei *et al.*, 2004; Matsushima *et al.*, 1998). Autophagy is induced in tumor cells transfected with the Nix homologue BNIP3 (Daido *et al.*, 2004).

Another mitophagic pathway in mammalian cells involves the ubiquitin-ligase Parkin to direct the destruction of depolarized mitochondria (Youle and Narendra, 2011). Whereas in mammalian cells Parkin is selectively recruited to depolarized mitochondria, thereby serving as a marker for mitochondria destined for mitophagy (Narendra *et al.*, 2008), in *S. cerevisiae* the mitophagy receptor Atg32 is evenly distributed over mitochondrial tubules (Mao *et al.*, 2011). Parkin recruitment to damaged mitochondria (that have lost their membrane potential) is due to stabilization of PINK1, which in healthy mitochondria is constitutively imported but degraded by the PARL protease (Lazarou *et al.*, 2012; Narendra *et al.*, 2008, 2010a). Parkin ubiquitinates many mitochondrial proteins, including the mitofusins Mfn1 and Mfn2, thus preventing fusion of defective mitochondria with the functioning network (Narendra *et al.*, 2010b), though this by itself is insufficient for mitophagy (Twig *et al.*, 2008). The *S. cerevisiae* mitofusin Fzo1 undergoes ubiquitination by SCF^{Mdm30} (Cohen *et al.*, 2008) and SCF^{Mfb1} (Durr *et al.*, 2006). These ubiquitin ligases could be the functional equivalents of Parkin in mammalian cells, but their roles in yeast mitophagy have not been assessed.

MITOCHONDRIAL DYNAMICS AND MITOPHAGY

In most eukaryotic cells mitochondria form a dynamic network and undergo continuous fission and fusion. Disregulated fission or fusion reduces mitochondrial functionality (Parone *et al.*, 2008). Mitochondrial fusion promotes their repair and increases their capacity for respiration (Rambold *et al.*, 2011), whereas fission segregates damaged mitochondria for mitophagy (Abeliovich *et al.*, 2013; Mao *et al.*, 2013). Increased fusion is observed as a response to minor levels of mitochondrial stress, whereas increased fission occurs during periods of high stress. Starvation is an example of low stress, where mammalian cells increase mitochondrial fusion to protect their mitochondria from autophagy (Gomes *et al.*, 2011; Rambold *et al.*, 2011). The proteins involved in mitochondrial fusion and fission are members of the dynamin family. The first mitochondrial dynamin to be discovered was

named Mgm1 since in *S. cerevisiae*, mutation of it leads to a defect in mitochondrial genome maintenance (Jones and Fangman, 1992). Indeed, in mammalian cells loss of mitochondrial DNA (by treatment with ethidium bromide) leads to mitophagy (Luo *et al.*, 2013). The human homologue of MGM1 is named Opa1 due to its role in atrophy of the optic nerve (Alexander *et al.*, 2000; Delettre *et al.*, 2000). Mgm1 is found in two forms: one is an integral membrane protein anchored in the inner mitochondrial membrane, facing the intermembrane space, and the second a truncated version that is peripherally associated with the inner membrane (Wong *et al.*, 2000). The outer membrane possesses different fusion-promoting dynamins, termed mitofusins. *S. cerevisiae* has one mitofusin – Fzo1 (Hermann *et al.*, 1998; Rapaport *et al.*, 1998), and mammalian species have two partially redundant mitofusins – Mfn1 and Mfn2 (Chen *et al.*, 2003; Santel and Fuller, 2001). In contrast to the fusion machinery which has different proteins for inner and outer membranes, fission of both membranes is controlled by one protein in most metazoans (Drp1) and *S. cerevisiae* (Dnm1) where it was first identified (Bleazard *et al.*, 1999; Shin *et al.*, 1997). Although Dnm1 is cytosolic, it is recruited to mitochondria by adaptors such as Fis1 in both yeast (Zhang and Chan, 2007) and mammalian cells (Loson *et al.*, 2013).

The requirement of proteins promoting mitochondrial fission, such as Dnm1 or Fis1, for efficient mitophagy (Abeliovich *et al.*, 2013; Kanki *et al.*, 2009b; Mao *et al.*, 2013) suggests two potential scenarios: the first is that autophagosomes cannot engulf large pieces of mitochondria, and the second is that mitochondrial dynamics segregate components destined for mitophagy. In order to distinguish between these options, a *dnm1Δmgm1Δ* double mutant was used. Mgm1 is required for mitochondrial fusion (Sesaki *et al.*, 2003; Wong *et al.*, 2000, 2003); *dnm1Δmgm1Δ* cells have normal mitochondrial morphology but lack mitochondrial dynamics. If size was important for mitophagy, then it would be expected for these cells to be more proficient in mitophagy than *dnm1Δ* cells. However, if mitochondrial dynamics are important for mitophagy, then despite a normal mitochondrial size, *dnm1Δmgm1Δ* cells would have similar mitophagy kinetics to *dnm1Δ* cells. Indeed, *dnm1Δmgm1Δ* cells have similarly reduced mitophagy as *dnm1Δ* cells suggesting that mitochondrial dynamics, not size, determine the rate of mitophagy (Abeliovich *et al.*, 2013).

Interestingly, not only do mitochondrial dynamics permit the degradation of mitochondria by mitophagy; this also affects the identity of mitochondrial proteins targeted to the vacuole, with some such as Hsp78 being inefficiently targeted for mitophagy (Abeliovich *et al.*, 2013). It has been suggested that weak physical segregation coupled with mitochondrial dynamics may cause a "distillation" of defective proteins into mitophagy destined mitochondrial fragments. Recently, Mao *et al.* provided a direct molecular link between mitochondrial fission and mitophagy: they found that Dnm1 is selectively recruited to mitochondria (Mao *et al.*, 2013). Upon induction of mitophagy by starvation (YPL > SD-N) some of the Atg32 rapidly associates with Atg11 although the relationship, under this experimental regime, of mitophagy with mitochondrial quality control, is unclear. Mitophagy that is induced by administration of rapamycin to respiring cells does not show a requirement for mitochondrial dynamics (Mendl *et al.*, 2011), and the mechanistic reason for this difference remains to be uncovered.

In mammalian cells, mitochondria periodically fuse and then undergo asymmetric fission, with the daughter mitochondria frequently having different membrane potentials (Twig *et al.*, 2008). This phenomenon could delineate a sorting mechanism to segregate

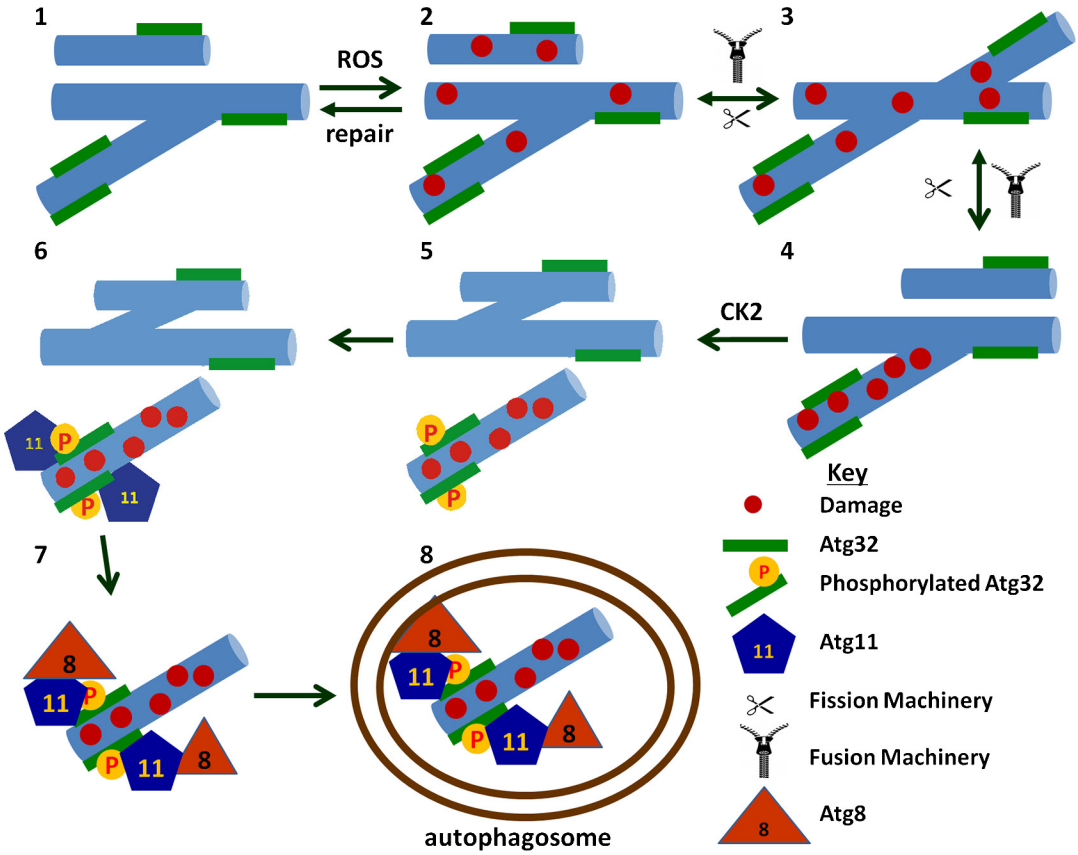

FIGURE 4.1 A model for mitophagy in *S. cerevisiae*. 1. When grown in respiratory medium, mitochondria undergo constant fission and fusion. Atg32 is distributed evenly around the mitochondrial reticulum. 2, 3, 4. Mitochondrial dynamics mediate the redistribution of defective components into segregated compartments. 5. Atg32 is phosphorylated by CK2. In this model we are assuming that only the Atg32 present on damaged mitochondria is phosphorylated, and this marks damaged mitochondria. Phosphorylated Atg32 recruits Atg11 to the mitochondrion. 6. Atg11 is recruited to the segregated, damaged compartment. 7. Atg11 recruits Atg8, thus commencing engulfment of the mitochondrion into the preautophagosome. 8. The damaged mitochondrion is enclosed in an autophagosome and is transported to the vacuole for degradation.

defective components (Abeliovich, 2011; Youle and van der Bliek, 2012). Indeed, evidence has recently been presented in favor of such a hypothesis in *S. cerevisiae* (Abeliovich *et al.*, 2013). Mitochondria that fail to recover their membrane potential are then targeted for autophagic degradation by PINK1 and Parkin (Narendra *et al.*, 2010a). Mitophagy in mammalian cells has also been reported to require Drp1 dependent mitochondrial fission (Frank *et al.*, 2012; Park *et al.*, 2012), suggesting that the role of mitochondrial dynamics in mitochondrial quality control is conserved. A schematic diagram of how the authors view the current understanding of yeast mitophagy is presented as Figure 4.1.

FUTURE DIRECTIONS OF YEAST MITOPHAGY RESEARCH

Although an impressive number of proteins involved in mitophagy have been identified, there remain a number of crucial questions in this field.

How is selectivity achieved? Whereas the mechanism downstream of Atg32 is well defined, Atg32 is homogeneously distributed in the mitochondrial network, and therefore its levels *per se* cannot impart selectivity to the process. Post-translational modifications of Atg32 must hold the key to understanding selectivity. Very little is known about the manner in which regulation of selectivity is achieved during mitophagy. Though there is ample evidence for ROS being a cause of mitophagy, a mechanism by which ROS-damaged mitochondria trigger their own selective degradation (while bulk-mitochondria remain active) has not been determined. The nature of the damage to mitochondria, how this damage is sensed to cause select recruitment of damaged mitochondria to autophagosomes, and what are the signaling pathways involved remain a mystery. Recent discoveries, such as the involvement of MAP kinase pathways and CK2, though providing some partial answers, still lack obvious stimuli to induce or inhibit mitophagy. Since these kinases are involved in signaling in many processes, the question of prevention of inappropriate mitophagy induction needs to be addressed.

Cells possess a range of solutions to the problem of mitochondrial stress, from RTG pathway activation, proteases, protein quality control, and mitophagy. However, we currently do not possess an understanding of the mechanism (or mechanisms) that coordinates these responses and activates them in an interdependent, hierarchical fashion.

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CHAPTER

5

Mitophagy: An Overview

Dalibor Mijaljica, Mark Prescott, and Rodney J. Devenish

OUTLINE

Abstract

Mitochondria play an important role in a number of processes that are essential to the life of cells as well as being key players in cell death mechanisms. The removal (elimination/degradation) of mitochondria through a selective autophagy, known as mitophagy, is critical for maintaining proper metabolic function and control at both the mitochondrial and cellular levels. Mitophagy can be triggered by diverse signals in the context of either a specific physiologic requirement for the removal of mitochondria (e.g., maturation of erythrocytes), or in disease situations in order to limit the effects arising from the presence of damaged mitochondria. In this chapter, we provide an overview of recent progress in understanding of the mechanisms of mitophagy and their regulation by considering both physiological and pathological roles of mitophagy including development, aging, cancer, neurodegenerative diseases, infection, and inflammation.

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SNAPSHOT: MITOPHAGY

Mitophagy is the selective degradation of mitochondria by delivery to the lysosome/vacuole utilizing autophagic processes. It is of considerable interest to both life scientists and clinicians because of the many novel aspects of the pathway and the links between (dys) function of mitophagy and physiological processes (e.g., development and aging) or certain diseases (e.g., neurodegeneration, cancer and immunity) (Ding and Yin, 2012; Jin *et al*., 2013; Taylor and Goldman, 2011).

Much of the information concerning the mechanism, regulation, and selectivity of mitophagy comes principally from studies conducted in the single cell eukaryote baker's yeast, *Saccharomyces cerevisiae* (Bhatia-Kiššová and Camougrand, 2012; Jin *et al*., 2013) or cultured mammalian cells (Ashrafi and Schwarz, 2012; Goldman *et al*., 2010). These studies reveal that engulfment of mitochondria largely occurs by autophagosome formation (macroautophagy or specifically macromitophagy). In yeast, engulfment of mitochondria can also occur by a mechanism involving direct interaction of the mitochondrion with the vacuolar membrane (microautophagy or specifically micromitophagy) and without the need for an autophagosome. Macromitophagy, commonly referred to as mitophagy, is the best characterized form of mitophagy and the form most commonly reported in the literature. Mitophagy has been observed to occur under three broad categories of circumstance: developmental changes, basal, and stress-induced. The first category includes the roles of mitophagy in cellular processes such as stem cell maintenance and differentiation (Vazquez-Martin *et al*., 2012), erythrocyte maturation (Novak and Dikic, 2011), and removal of paternal mitochondria (Sato and Sato, 2011). The second category includes normal physiological conditions under which mitophagy plays a role in the processes of cellular function, and homeostatic quality control whether the organism be single-cell or multicellular (Bhatia-Kiššová and Camougrand, 2012; Goldman *et al*., 2010). The third category consists of occurrences of mitophagic activity that take place in response to damaged or dysfunctional mitochondria, or in the case of yeast significant change in cellular metabolic context (e.g., nutrient deprivation or change in carbon source), resulting in the targeted and selective engulfment of those mitochondria and their subsequent degradation (Bhatia-Kiššová and Camougrand, 2012; Ding and Yin, 2012; Jin *et al*., 2013; Taylor and Goldman, 2011).

Molecular Mechanisms and Regulation

Mitophagy utilizes the same core autophagy machinery (largely encoded by *Aut*ophagy-related *G*enes, *ATG* genes) as do other forms of selective autophagy (Jin *et al*., 2013), whether induced by different intrinsic cues (e.g., genetically programmed versus damage occasioned by cellular metabolism) or extrinsic factors (e.g., environmental stimuli) (Bhatia-Kiššová and Camougrand, 2012; Ding and Yin, 2012; Jin *et al*., 2013; Taylor and Goldman, 2011).

There is considerable interest in gaining an understanding of the pathways that regulate mitophagy in different cells and tissue types, and the strategies used to ensure selective targeting of the autophagic machinery towards mitochondria. The exact molecular mechanisms and signaling pathways behind the regulation of mitophagy are only now beginning to be revealed. A cargo-ligand-receptor-scaffold model has been proposed to account for how cells can select between different cargo such as the mitochondrion for degradation

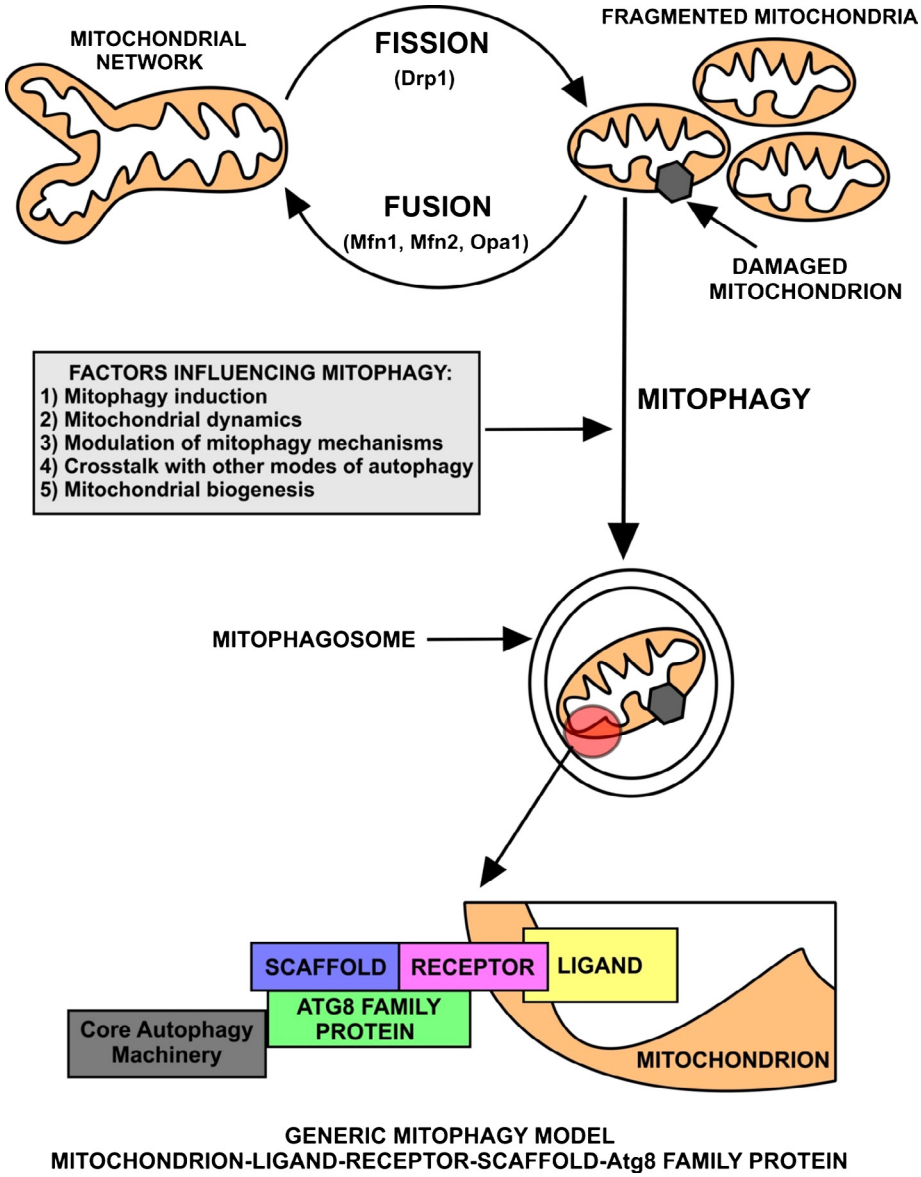

FIGURE 5.1 Mitochondrial dynamics and a molecular model for mitophagy. Under normal conditions, mitochondria constantly undergo cycles of fission and fusion, which helps preserve proper mitochondrial function through mixing of matrix contents. An imbalance in the fission/fusion equilibrium or mitochondrial damage can lead to degradation of mitochondria by mitophagy. Interrelated factors such as mitophagy induction, mitochondrial dynamics (fission and fusion), modulation of mitophagy molecular mechanisms by targeting individual components, crosstalk with other modes of autophagy, and mitochondrial biogenesis form a complex interacting network that governs mitochondrial degradation by mitophagy and, thereby, mitochondrial function and cellular integrity. A challenge for future studies is to determine the molecular details of how these factors may regulate mitophagy and develop nodes where intervention specific for mitophagy will lead to clinically useful outcomes. A generic model of the mitochondrion-ligand-receptor-scaffold-Atg protein model required for mitophagy is shown. The mitochondrion-ligand-receptor-scaffold associates with the forming mitophagosomal membrane and interacts with the core autophagy machinery. Candidate proteins for each of the components in the model are discussed in the main text.

(Figure 5.1) (Jin *et al.*, 2013; Mijaljica *et al.*, 2012). The identity of each of the ligand-receptor-scaffold components appears to differ depending on the organism, cell-type, and mitophagy stimulus. In yeast, mitophagy can be associated with the cellular remodeling that occurs upon the transition of cell growth and metabolism to a preferred carbon source. For example, when yeast cells are shifted from respiratory substrates such as lactate to glucose, excess mitochondria are degraded. The mitochondrial outer membrane (MOM) protein Atg32 functions as a receptor for mitophagy and interacts with Atg11 (scaffold protein) and Atg8 (autophagosome membrane-anchored protein) (Jin *et al.*, 2013). Atg32 appears to be the sole receptor for mitophagy in yeast although proteins such as Atg33 can facilitate the process. Phosphorylation events play a key role in regulation of mitophagy. An as-yet unidentified kinase phosphorylates Ser114 and Ser119 on Atg32; the former mediates the Atg11–Atg32 interaction (Hirota *et al.*, 2012).

In mammals, the functional counterpart of Atg8 is microtubule-associated protein 1 light chain 3 (LC3) and while the counterpart of Atg32 has yet to be found, evidence exists for a number of different proteins that are able to function as receptors to link mitochondria with the autophagy machinery, depending on the cell type. Bcl-2/Adenovirus E1B 19 kDa protein-interacting protein 3-like (BNIP3L), also called Nix, is a member of the Bcl-2 family of apoptotic regulators. BNIP3L translocates to mitochondria and is required for mitophagy during erythroid differentiation. Ectopic expression of BNIP3L causes activation of mitophagy even in normoxia (Novak and Dikic, 2011). Echoing the importance of phosphorylation events in yeast mitophagy, in hypoxia the dephosphorylation of FUNDC1 (FUN14 domain containing 1), an integral mitochondrial outer-membrane protein, enhances its interaction with LC3, via an LC3 interacting region (LIR) motif, to induce mitophagy. Under normal physiological conditions, FUNDC1 is phosphorylated at Tyr18 located within the LIR motif by Src kinase and mitophagy is inhibited. Interestingly, although both Nix and FUNDC1 are involved in mitophagy induced by hypoxia their roles in the induction of mitophagy appear different (Liu *et al.*, 2012).

Mitochondrial outer membrane proteins are ubiquitinated in depolarization-induced mitophagy (see the following). SQSTM1 (sequestosome 1/p62) contains an LIR motif allowing it to function as an adaptor that links LC3 to ubiquitinated proteins associated with (embedded or on) the outer mitochondrial membrane to the core proteins of the autophagy machinery (Jin *et al.*, 2013).

Mitochondrial Dynamics and Mitophagy

Mammalian mitochondria form a highly dynamic reticular network constantly undergoing fusion and fission events, with the relative extent of these two events influencing mitochondrial quality control (Figure 5.1). Fusion is mediated by mitofusins 1 and 2 (Mfn1, Mfn2) and optic atrophy 1 (OPA1), which have roles in fusion of the outer and inner membranes of the mitochondrion, respectively (Scott and Youle, 2010; Twig and Shirihai, 2011). The dynamin-related protein 1 (Drp1) is essential for fission and is regulated through phosphorylation by PKA and Cdk1/cyclin B (Cribbs and Strack, 2007). A regulatory role for phosphorylation in fusion has been suggested (Schauss *et al.*, 2010), but no targets have been identified.

Fusion promotes intermixing of mitochondria and contributes to the integrity and homogeneity of the mitochondrial network, protects against loss of mitochondrial DNA (mtDNA),

and supports an optimal bioenergetic activity. On the other hand fission promotes the distribution and inheritance of mitochondria and helps in isolation of the dysfunctional (damaged) mitochondria from the intact network and their selective removal by mitophagy (Escobar-Henriques and Anton, 2013). Indeed, in mammalian cells mitophagy is impaired when mitochondrial fission is blocked and dysfunctional mitochondria accumulate. Several neurodegenerative diseases such as Parkinson's disease are associated with alterations in mitochondrial dynamics (Müller and Reichert, 2011).

Monitoring Mitophagy

The presence of mitochondria within autophagosomes (also known as mitophagosomes, autophagosomes specifically and selectively containing mitochondria) arising from mitophagic activity in cells was first observed by electron microscopy (EM) in mammalian cells and later in yeast (Klionsky *et al*., 2012). EM remains a primary technique for investigation of mitophagy since it offers the potential to confirm the cargo as mitochondria as well as yield information on membrane interactions contributing to mitophagy. However, more facile analytical approaches are routinely used to monitor mitophagy in mammalian cells (and other models). These include specific imaging techniques such as fluorescence microscopy, as well as following protein degradation in cell lysates by western immunoblotting. Using the latter approach the levels of a specific protein can be followed in protein lysates prepared from cells under different conditions and/or treated with autophagy inducers/inhibitors to monitor flux through the system. A caveat associated with this approach is that recent evidence suggests that individual mitochondrial proteins/compartments may be handled differently by mitochondrial quality control systems including mitophagy and, therefore, the fate of more than one mitochondrial protein should be followed. Using fluorescence microscopy, events in individual cells can be followed. Most commonly, LC3 fused with a fluorescent protein (e.g., GFP-LC3) serves as the marker of the autophagosome and can be coupled with use of a fluorescent dye (e.g., Mitotracker Red) to specifically label mitochondria. Co-localization of the two fluorescent signals would be interpreted as indicating mitophagy. Alternatively, labeling of mitochondria with a fluorescent biosensor such as Rosella (a fusion of the relatively pH-stable fast-maturing RFP variant, DsRed.T3 with a pH-sensitive GFP variant, pHluorin) allows delivery of mitochondria to the acidic lysosome to be followed by exploiting its unique pH-sensitive dual emission properties (Klionsky *et al*., 2012; Mijaljica *et al*., 2011).

It should be noted, however, that both these methods reveal less information concerning the mechanism of mitophagy. It is necessary to demonstrate and monitor the steady-state levels of mitophagy by a combination of assays yielding the following types of information: (1) increased levels of mitophagosomes, (2) maturation of mitophagosomes culminating with mitochondrial degradation, which can be blocked by specific inhibitors of autophagosomal formation or of lysosomal degradation, and (3) whether the changes are due to selective mitophagy or increased mitochondrial degradation during generalized autophagy. Techniques and assays to address each of these points have been extensively reviewed (Klionsky *et al*., 2012). In pathophysiological situations, in addition to monitoring the steady-state levels of different steps of mitophagy, investigation of the mitophagic flux is needed to decipher which steps within the process fail to result in efficient mitochondrial degradation (Klionsky *et al*., 2012).

PHYSIOLOGICAL AND PATHOLOGICAL ROLES OF MITOPHAGY

As remarked upon above, mitochondria are not static organelles but typically form a dynamic network that is constantly remodeled by fission/fusion events (Figure 5.1) in concert with changes in rates of biogenesis and degradation. As organelles they are integrated into the cellular signal transduction network, responding to both signals from outside the cell and internally generated either outside or within a mitochondrion. Therefore, alterations in mitochondrial fission/fusion dynamics, biogenesis or degradation, as well as disorders of mitochondrial metabolic related functions related to their essential role in processes such as oxidative phosphorylation or fatty acid oxidation, lead to a large variety of diseases. The disease outcomes can manifest in many organs and tissues, and particularly in cell types that have a high energy demand and, therefore, reliance on mitochondrial function such as neuronal cells. Thus, mitophagy has now been shown to contribute to a multitude of physiological processes, including development and aging as well as pathological conditions such as cancer, neurodegenerative diseases, bacterial and viral infections and inflammation (Taylor and Goldman, 2011). In the following sections, we will briefly overview the contribution of mitophagy to some of these physiological processes and mitophagy-related diseases.

Development

Developmental processes such as reticulocyte maturation (Novak and Dikic, 2011), and elimination of paternal mitochondria during early embryogenesis (Sato and Sato, 2011) entail mitophagy of surplus but otherwise healthy mitochondria in a programmed manner. Here removal of mitochondria is essential for successful tissue and organ development. As indicated previously, BNIP3L/Nix is indispensable for elimination of mitochondria during reticulocyte maturation. The loss of mitochondria and other organelles such as the nucleus allow the highly specialized erythrocytes to accommodate maximal levels of hemoglobin, thereby maximizing oxygen transfer from lungs to the peripheral tissues. BNIP3L/Nix knockout mice retain mitochondria in erythrocytes and consequently develop anemia and severe reticulocytosis caused by inefficient loss of mitochondria, resulting in decreased survival of these cells (Palikaras and Tavernarakis, 2012). BNIP3L/Nix acts as a mitophagy receptor such that when mitochondria are depolarized, it influences translocation of Parkin to mitochondria, which in turn ubiquitinates mitochondrial proteins, thereby marking mitochondria for mitophagy. BNIP3L is involved in hypoxia-induced mitophagy acting as a mitophagy receptor through the same basic autophagic mechanism. Thus, BNIP3L/Nix acts as a mitophagy receptor under different conditions, during cellular differentiation (in reticulocytes) and in stress conditions such as hypoxia. It is not yet clear whether BNIP3L/Nix can be utilized when in terminally differentiated cells when damaged mitochondria need to be removed to protect cells from further damage and/or cell death (Novak and Dikic, 2011).

Another important developmental role for mitophagy is the removal of paternal mitochondria in fertilized oocytes. The mitochondrial genome is maternally inherited in mammals and some other multicellular organisms. Sperm-derived paternal mitochondria enter the oocyte cytoplasm upon fertilization and under normal circumstances are removed

during early embryogenesis. Until recently little was known about the mechanism responsible for this clearance. Two studies using *Caenorhabditis elegans* have shown that, immediately after fertilization, sperm-derived components trigger the localized induction of mitophagy of sperm-derived mitochondria. Autophagosomes engulf the paternally derived mitochondria, resulting in their lysosomal degradation during early embryogenesis. In autophagy/mitophagy-defective zygotes, paternal mitochondria and their genome persist during development and can be found in the first larval stage. Thus, fertilization-triggered autophagy/mitophagy is required for selective degradation of paternal mitochondria, thereby ensuring the maternal inheritance of mtDNA. In mice, autophagy (and possibly mitophagy) is upregulated immediately after fertilization, suggesting that fertilization-triggered autophagy is a conserved phenomenon in mammals. However, the signals that activate selective elimination of sperm-derived mitochondria and its regulation remain unknown (Sato and Sato, 2011).

Aging

The fission and fusion of mitochondria help maintain a healthy population of mitochondria through mixing of mitochondrial contents (of principal importance would be mtDNA) when the extent of molecular damage is low. However, the levels of molecular damage that accumulate during the aging process make it ineffective as a way of dealing with damage (i.e., accumulated mtDNA mutations). It is more beneficial for a cell to maintain a healthy mitochondrial population by isolating damaged organelles for subsequent mitophagy. A reduction in the number of fusion–fission cycles, however, makes the cellular population of mitochondria more vulnerable to newly acquired random molecular damage (Figge *et al.*, 2013). Presumably this is the case because there is less mixing of mitochondrial contents and, therefore, damage is more "isolated." Furthermore, the formation of enlarged or highly interconnected mitochondria as a result of increased fusion and/or decreased fission events can diminish mitochondrial turnover by impairing mitophagy, leading to the accumulation of damaged mitochondria in aged cells (Seo *et al.*, 2010). This situation arises because the fission of the mitochondrial network generates some smaller damaged mitochondria which are recognized as the target for mitophagy. Clearly, age-associated alterations in mitochondrial fusion and fission dynamics have the potential to play a causative role in mitochondrial dysfunction, and increase susceptibility to cell death in response to various types of stress, particularly from *R*eactive *O*xygen *S*pecies (ROS) during progressive aging (Figge *et al.*, 2013; Taylor and Goldman, 2011).

The exact mechanism by which damaged mitochondria are selected from the mitochondrial pool, thereby facilitating their removal and consequently delaying aging, is not fully clear. It is now generally accepted that mitophagy contributes to a reduction in the production of mitochondria-derived ROS (mtROS) such as nitroxides, hydrogen peroxide, and superoxide anions, and remove dysfunctional/damaged mitochondria and/or mitochondrial proteins that would generate mtDNA mutations (including point mutations and deletions) during aging (Figge *et al.*, 2013). Studies on aging in humans and animal models have established a correlation between aging, a decline in mitochondrial function, and the accumulation of mtDNA mutations (Taylor and Goldman, 2011). Oxidative insults

to mitochondria, in turn, impair the primary functions of the mitochondria such as energy transduction, biogenesis of metabolites, Ca^{2+} homeostasis, and regulation of redox-biology with age, thereby contributing to a vicious cycle of accumulating mitochondrial damage that culminates in a mitochondrial functional crisis. This crisis ultimately results in cell death and aging (Seo *et al.*, 2010). Caloric restriction is known to promote longevity from yeast to mammals. Given that caloric restriction induces autophagy, increased longevity may in part originate from enhanced elimination of dysfunctional mitochondria. Further studies should clarify whether mitophagy is indeed involved in mediating part of the effects of caloric restriction on lifespan (Palikaras and Tavernarakis, 2012).

Cancer

A reduction in autophagic capacity occurs as we age, thereby contributing to the accumulation of damaged cellular components and possibly tumor development (Taylor and Goldman, 2011). This idea is supported by the observation that the phenotype described for the autophagy deficient mouse (Beclin $1^{-/-}$) included a high rate of spontaneous tumor development (Qu *et al.*, 2003). Moreover it was later noted that *Beclin 1* monoalleleic deletion on chromosome locus 17q21 occurs in 40–70% of human ovarian, breast, and prostate cancers (Taylor and Goldman, 2011). Mice with allelic loss of *Beclin 1* are prone to hepatocellular carcinoma, lung adenocarcinoma, mammary hyperplasia, and lymphoma (Ding and Yin, 2012). In this context the link between autophagy and cancer is the role of genes encoding key autophagy proteins as tumor suppressors. This is illustrated by the observations that mice with liver-specific loss of the Atg7 or Atg5 protein accumulate damaged mitochondria and develop liver injury, steatohepatitis, and adenocarcinoma (Ding and Yin, 2012). Additional studies have shown that autophagy deficient tumor cells accumulate damaged mitochondria, ROS, and protein aggregates, all of which may contribute to tumor development and progression. It is probable that an association between a loss of autophagic (in general) and mitophagic (specifically) capacity and an increased risk of cancer development exists; however, exactly how autophagy acts to mitigate the damage associated with tumor formation is still not fully understood (Taylor and Goldman, 2011). Other lines of evidence from human tumors and cancer cell lines have suggested that specific somatic mtDNA mutations are associated with tumor growth. In addition, many types of mtDNA alterations have been identified in cancer cell lines, suggesting the possibility that damaged mitochondria and reduced ability for their mitophagic elimination may contribute to oncogenesis. Taken together, these observations imply that mitophagy may act as a tumor suppression mechanism via the removal of damaged mitochondria (Taylor and Goldman, 2011). For example, mitophagy may act to buffer metabolic stress caused by limited nutrients or oxygen in the tumor tissues (Ding and Yin, 2012). Future work is needed to further determine the exact contribution of mitophagy in tumorigenesis.

Neurodegenerative Diseases

Terminally differentiated, nonmitotic cells such as neurons require multiple protection systems in order to prevent their untimely death. Since neurons have such high energy demands they rely heavily on proper functioning of their mitochondrial network. In

addition, they rely on mitophagy to maintain a healthy mitochondrial population due to constant bombardment by self-generated ROS (mtROS) that can further impair mitochondrial function and have devastating effects on the integrity of mitochondria and ultimately cells. The close relationship between mitochondrial function and dynamics, suggests that in neurons, alterations in function may be exacerbated by changes in mitochondrial fission and fusion, thereby contributing to neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), and Huntington's disease (HD) (Palikaras and Tavernarakis, 2012; Taylor and Goldman, 2011).

In PD, upon mitochondrial dysfunction arising as a consequence of mitochondrial membrane depolarization, PINK1 (PTEN-induced kinase 1/PARK6) is selectively stabilized on the MOM. Normally PINK1 is transported across the MOM and processed in a membrane potential-dependent manner to maintain mitochondrial function and to suppress mitophagy. Upon mitochondrial depolarization this processing is arrested so that PINK1 is exposed on the MOM where it accumulates and leads to the recruitment of PARK2/Parkin, an E3 ubiquitin ligase, from the cytoplasm. Following recruitment of Parkin, PINK1 kinase activity results in enhanced ubiquitin ligase activity of Parkin (Kawajiri *et al*., 2010; Matsuda *et al*., 2010) which in turn leads to the formation of poly-ubiquitin chains on MOM proteins including the mitofusins, MFN1 and MFN2, and VDAC1 (Youle and van der Bliek, 2012). This in turn triggers recruitment of SQSTM1/p62 to mitochondria (see above) initiating mitophagy (Jin *et al*., 2013).

Recent work has indicated that autosomal recessive mutations in PINK1 and Parkin lead to familial forms of PD, presumably via failure of mitophagy, which contributes to disease pathogenesis. Alternatively, it is possible that PINK1 dysfunction promotes excessive turnover of healthy mitochondria. Also in these contexts PINK1 and Parkin function likely intersects with the process of mitochondrial dynamics, and vice versa (Batlevi and Spada, 2011).

Studies in the fruit fly, *Drosophila melanogaster*, have linked Drp1-mediated mitochondrial fission with the Parkin and PINK1 mutations that cause PD in humans. These studies indicate that action of PINK1 and Parkin is directed towards maintaining mitochondrial quality control, as increased Drp1 expression in Parkin or PINK1 mutant flies can rescue mitochondrial defects, while decreased Drp1 dosage in combination with Parkin or PINK1 mutation is lethal. Although one study of PINK1 in mammalian systems has linked PINK1-dependent mitophagy to mitochondrial fission, the role of Parkin and PINK1 in mitochondrial dynamics, and the nature of the cross-talk between mitochondrial dynamics and mitophagy in the mammalian nervous system, remain ill-defined (Batlevi and Spada, 2011).

Alzheimer's disease patients possess damaged mitochondria; in particular, mitochondrial cytochrome oxidase is defective. Accumulation of β-amyloid fragments within the mitochondria has been proposed as providing a mechanistic insight into mitochondrial dysfunction in AD. No direct evidence exists for a role of mitophagy in ameliorating the disease (Batlevi and Spada, 2011). Recently, progress has been made in the interpretation of the connection between mitochondrial dynamics and mitophagy with regard to AD. It was demonstrated that there exists an imbalance between mitochondria fission/fusion in fibroblasts from AD patients and in a neuroblastoma cell line over-expressing a variant of β-amyloid (Taylor and Goldman, 2011). The fibroblasts exhibited increased mitochondrial fusion resulting in elongated mitochondria, the inhibition of efficient mitophagy, and the promotion of damage presumably due to an inability to dispose of the dysfunctional mitochondria.

By contrast, the neuroblastoma cell line exhibited excess fission, possibly contributing to altered mitochondrial function, particularly increased ROS production, and the preoccupation of mitophagic machinery with the removal of damaged mitochondria, thus preventing the efficient removal of other autophagic cargo such as protein aggregates to the overall detriment of the cell (Taylor and Goldman, 2011). Furthermore, it was demonstrated that the inhibition of autophagy in neurons was followed by accumulation of β-amyloid protein aggregates, implying that autophagy normally acts to prevent the formation of these aggregates (Taylor and Goldman, 2011). In addition, two different neuron-specific autophagy knockout cell lines (*ATG5* $^{-/-}$ and *ATG7* $^{-/-}$) exhibited an increase in protein aggregate formation, emphasizing the importance of autophagy (and possibly mitophagy) in the clearance of normal cytosolic proteins (Taylor and Goldman, 2011).

In HD, transcriptional dysregulation of the peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), which has an important role in several metabolic processes including mitochondria biogenesis and oxidative phosphorylation, might be a contributing factor to HD pathogenesis. Importantly, metabolic and transcriptional defects in HD mice and patients can be linked to dysregulation of PGC-1α (Batlevi and Spada, 2011), highlighting the importance of mitochondrial physiology in HD pathogenesis. Furthermore, different studies have shown that decreased mitochondrial membrane potential, defects in mitochondrial Ca^{2+} uptake, decreased respiratory function, reduced mitochondrial mobility, and changes in mitochondrial structure as well as reduced mitochondrial fusion and increased mitochondrial fragmentation are some of the observed mitochondrial defects in HD patients (Palikaras and Tavernarakis, 2012). It is possible that mitophagy is a defense against neuronal loss in HD by the elimination of defective mitochondria, thereby preventing the caspase activation that has been identified in HD and linked to huntingtin protein (Htt) cleavage (Batlevi and Spada, 2011). Also, alterations in mitochondrial fusion and fission induce macroautophagy (and possibly mitophagy) to favor their removal (Twig *et al.*, 2008).

Infection and Inflammation

Inflammation cannot be thought of solely as a negative contributor to disease. It is an important physiological response against invading pathogens (bacteria and viruses) and contributes to repairing damage and preventing further tissue or cell injury. Mitochondria modulate innate immunity through redox-sensitive inflammatory pathways by direct activation of inflammasomes such as NLRP3 (NOD-, LRR- and pyrin domain-containing 3). Mitochondrial damage-associated molecular patterns (DAMPs) can trigger mitophagy. DAMPs are released by stressed cells undergoing autophagy or injury, and act as endogenous danger signals to regulate the subsequent inflammatory and immune response (Zhang *et al.*, 2013). In this manner inflammation is controlled by inhibiting NLRP3 inflammasome activation through the removal of ROS-producing mitochondria. Inhibition of mitophagy results in inflammasome activation (Nakahira *et al.*, 2011). Furthermore, decreased expression of genes encoding autophagy/mitophagy proteins, as well as mutations in these genes, has been reported for different diseases having an inflammation component as well as in aging. For example, the core autophagy/mitophagy related genes *ATG5* and *ATG12* have been shown to inhibit RIG-I like receptors (RLR). RLRs signal through interferon-β promoter stimulator 1 (IPS-1), resulting in the production of the key antiviral cytokines, type I interferons.

Autophagy/mitophagy could regulate the level of RLR signaling in two nonmutually exclusive ways. First, those autophagy/mitophagy deficient cells may produce more interferons and cytokines upon RLR stimulation as a result of an increase in the number of mitochondria per cell, leading to IPS-1 accumulation on a per-cell basis. Second, enhanced RLR signaling in the absence of autophagy/mitophagy may result from the accumulation of dysfunctional mitochondria that harbor elevated levels of ROS (Cloonan and Choi, 2012). ROS have been shown to activate starvation-induced autophagy and antibacterial autophagy and a number of Toll-like receptors (TLRs) have been reported to stimulate autophagy in phagocytes. Recent evidence suggests that suppression of autophagy/mitophagy leads to the accumulation of damaged, ROS-generating mitochondria, and this in turn activates the NLRP3 inflammasome. This notion is based on the following observations: (1) inhibition of complex I or III of the mitochondrial respiratory chain, known to result in ROS generation, causes unprompted NLRP3 inflammasome activation; (2) inhibition of mitophagy/autophagy, resulting in the prolonged presence of damaged, ROS-generating mitochondria, leads to spontaneous inflammasome activation; (3) NLRP3 and apoptosis-associated speck-like protein (ASC) co-localize with mitochondria and mitochondria-associated ER (endoplasmic reticulum) membranes in the presence of NLRP3 inflammasome activators; (4) knock down by shRNA or inhibition by Bcl-2 of voltage-dependent anion channels (VDAC) that are crucial in mitochondrial activity and ROS generation significantly impair NLRP3 inflammasome activation (Ding and Yin, 2012). Resting NLRP3 localizes to ER structures, whereas on inflammasome activation both NLRP3 and its adaptor protein ASC redistribute to the perinuclear space where they co-localize with ER and mitochondria organelle clusters. Notably, both ROS generation and inflammasome activation are suppressed when mitochondrial activity is dysregulated by inhibition of the VDAC. This indicates that NLRP3 inflammasome senses mitochondrial dysfunction and may explain the frequent association of mitochondrial damage with inflammatory diseases (Ding and Yin, 2012). Although autophagy has been implicated as a control mechanism for innate immune signaling in a number of other disease models (e.g., biliary cirrhosis in liver and PD) (Deretic and Levine, 2009), little is known about the role for mitophagy in such pathways. However, in biliary cirrhosis in liver, aberrant mitophagy in "stressed" biliary epithelia cells may initiate the immune response against mitochondrial antigens (Cloonan and Choi, 2012).

PREVENTIVE MEASURES FOR MITOPHAGY-RELATED DISEASES

The process of mitophagy is responsible for "quality control" degradation of mitochondria. As indicated above it is a complex process that is highly regulated under both physiological and pathological conditions. Rapid progress in our understanding of mitophagy mechanisms, their regulation, and connection to mitochondrial dynamics, as well as the pathology of mitochondria-related diseases offers the prospect of mitophagy-related therapies and treatments for various mitophagy-associated diseases (Figure 5.1).

Possible interventions targeted specifically towards mitophagy include the modulation of mitophagic activity through the following: (1) prevention of mitochondrial damage and associated elevated ROS production, bearing in mind, however, that ROS generation is also important in normal cellular signaling events (e.g., inflammation); (2) alteration

in the balance between mitochondrial fission and fusion (e.g., neurodegenerative diseases); (3) the alteration of the machinery responsible for the selectivity of mitophagy; and (4) the (in)direct induction and/or inhibition of mitophagy. Which of the approaches listed here will result in the most practical therapeutic interventions for mitophagy-related diseases remains to be seen, but it is exceedingly likely that some or all will be used in the near future (Figure 5.1) (Taylor and Goldman, 2011).

Importantly, we appreciate that mitochondrial fusion/fission is an important aspect of the mitophagic process. Given that normally fission and fusion are in "balance" commensurate with the demand for mitochondrial function in any particular tissue or cell type, then any treatment directed at this "target" would need to be directed at restoring that balance. A treatment regime directed towards decreased/impaired fusion or enhanced fission, which correlates with increased mitophagy, would need to ensure sufficient functional mitochondria are maintained to sustain cell survival and permit biogenesis of replacement healthy mitochondria. Furthermore, the number of different receptors may be the key control point to target mitophagy. Is there some degree of cell type specificity in receptor/adaptor pair(s) used? It may prove feasible to selectively modulate the function of specific receptor/adaptor pairs if more than one contributes to mitophagy in a particular disease scenario (Figure 5.1). If phosphorylation (as for Atg32) or other post-translational modification (e.g., acetylation) of these proteins is important for function, then this offers a possible method of pharmaceutical intervention.

FUTURE QUESTIONS AND CONCLUDING REMARKS

The contexts for mitochondrial dynamics and induction of mitophagy are likely to be the subject of intensive investigation for some time. It is likely mitophagy will be recognized as being significant in further cellular settings. Intriguingly, recent observations suggest that mitophagy may serve to drive selective turnover of mitochondria prior to and during the programming of somatic cells to induced pluripotent stem cells (iPSCs) (Vazquez-Martin *et al.*, 2012).

There remains much to be learned about the mechanisms and casual relationships in a variety of experimental and pathophysiological settings. One question is how extensive mitophagy might be in different circumstances. Already there is the claim that extensive mitophagy occurs only when general macroautophagic and specific Parkin-mediated mitophagy pathways are activated together (de Vries and Przedborski, 2012). Very recently it has been reported that Gp78, an ER-associated ubiquitin ligase, regulates mitochondrial fission/fusion and can induce mitophagy when mitochondria are experimentally depolarized (Fu *et al.*, 2013). This is especially intriguing in light of the very recent report that autophagosomes form at ER-mitochondria contact sites (Hamasaki *et al.*, 2013). Interestingly, the Gp78-induced mitophagy is Parkin-independent and has a selective requirement for Mfn1, but not for Mfn2 (Fu *et al.*, 2013).

Another recently reported and intriguing connection between the ER and mitophagy is the observation that MOM proteins, including FKBP38 and Bcl-2, are translocated from mitochondria to the ER presumably as a means of escaping degradation. The translocation was shown to depend on the ubiquitin ligase activity of Parkin. Furthermore,

the translocation of FKBP38 was reported as being essential for the suppression of apoptosis during mitophagy. The results thus show that not all mitochondrial proteins are degraded during mitophagy (Saita *et al.*, 2013).

The identification of further ubiquitin ligases that regulate fission/fusion and/or mitophagy will raise interesting questions concerning the potential cooperative activity between them, including whether their individual or combinatorial action might be specific to particular cellular conditions. Indeed it is important to note that we cannot be certain we fully understand the cellular activities of a well-characterized ubiquitin ligase such as Parkin. Furthermore, additional mitochondrial surface proteins that function as receptors marking mitochondria for mitophagy may yet be identified. Recent data have suggested a receptor function for ceramide which interacts directly with LC3 interaction following Drp1-dependent mitochondrial fission to induce mitophagy (Sentelle *et al.*, 2012).

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CHAPTER

6

Mitophagy Induction and Curcumin-Mediated Sonodynamic Chemotherapy

Chuanshan Xu and Wingnang Leung

OUTLINE

Abstract

Sonodynamic chemotherapy or sonodynamic therapy has shown potential in the management of malignant tumors. Emerging evidence has demonstrated that curcumin, isolated from a traditional Chinese herb *Curcuma longa*, has significant cytotoxicity and apoptotic induction upon ultrasound sonication. Flow cytometer analysis showed that ultrasound treatment in the presence of curcumin increased the level of intracellular reactive oxygen species and caused mitochondrial damage in cancer cells. Recently, our transmission electron microscopy observed more mitophagies in nasopharyngeal cancer cells treated by ultrasound sonication in the presence of curcumin, indicating mitophagy induction is an indispensable event in the mechanism of sonodynamic chemotherapy with curcumin on nasopharyngeal cancer.

INTRODUCTION

Nasopharyngeal cancer (NPC), a malignant tumor of the nasopharynx, threatens populations in Southeastern Asia (Xu and Leung, 2006). In Hong Kong, nasopharyngeal cancer is one of the most common malignant tumors, accounting for 3.5% of all new cancer cases

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and 2.4% of all cancer deaths. The 2010 statistical report from the Department of Health of Hong Kong shows that NPC has become the ninth most common fatal cancer in the Hong Kong region (Department of Health of Hong Kong, 2012). The conventional therapies including surgery, radiotherapy, and chemotherapy have had many significant advances, but cancer recurrence and severe side effects are still the main limitation of these conventional therapies. Therefore, there is an urgent need to seek novel, safe, and highly efficient strategies for treating patients with nasopharyngeal cancer.

ALTERNATIVE THERAPEUTIC STRATEGIES: ULTRASOUND THERAPY AND SONODYNAMIC CHEMOTHERAPY

Photodynamic chemotherapy (PDCT) or photodynamic therapy (PDT) is a promising alternative for nasopharyngeal cancer. This therapeutic modality is based on the production of cytotoxic reactive oxygen species (ROS) from photosensitive drugs activated by appropriate wavelengths of harmless visible light, which damages the cell membrane system and oxidizes intracellular biomolecules, such as nuclear acid and proteins, subsequently causing cell death (Dougherty *et al*., 1998; Calzavara-Pinton *et al*., 2007; Pachydaki *et al*., 2007). Nowadays, PDT as an alternative has been widely used in clinical treatment of malignant tumors (Baldea and Filip, 2012). Growing studies have shown that PDT also effectively kills nasopharyngeal cancer (Xu and Leung, 2006). Interestingly, recent studies found that high-intensity ultrasound can directly cause damage to tumor cells and low-intensity ultrasound can activate many sensitizers to produce cytotoxic ROS to kill tumor cells. High-intensity focal ultrasound (HIFU) is a well-known method that uses high temperature produced by high-intensity ultrasound in target tissues to damage tumor cells and tissues. Clinical trials demonstrated that HIFU showed promise in treating pancreatic cancer, liver cancer, prostate cancer, renal cancer, bone tumor, breast cancer, etc. (Dutcher *et al*., 2012; Orgera *et al*., 2011; Orsi *et al*., 2010; Ryan *et al*., 2012; Wang, K. *et al*., 2012; Wijlemans *et al*., 2012). Low-intensity ultrasound has potential in enhancing the efficacy of chemotherapeutic drugs. Investigators reported that low-intensity ultrasound treatment increased intracellular concentration of anticancer drugs, enhanced the killing action and the inhibitory activity of drugs on the adhesion and migration of cancer cells (Zhou *et al*., 2011). These findings indicate the combination of low-intensity ultrasound and chemotherapeutic drugs might be a potential strategy in the management of malignant tumors.

With the advances of ultrasound therapy in the medical field, researchers reported low-intensity ultrasound waves could also activate photosensitizers to produce ROS to kill tumor cells, termed sonodynamic therapy (SDT) (Misík and Riesz, 2000) or sonodynamic chemotherapy (SDCT). The main principle of the action of sonodynamic therapy on a tumor is dependent on sonochemical action through the interaction of low-intensity ultrasound and sensitizer in the presence of molecular oxygen inside tumor tissues – for example, the activated sensitizer directly interacts with molecular oxygen to generate a kind of ROS, namely singlet oxygen ($^1O_2$), which oxidizes lipid membranes, enzymes, and nucleic acid to damage tumor cells. Furthermore, sonochemical action also produces free radicals, which interact with molecular oxygen to produce ROS to cause lethal damage to tumor cells and tissues (Misík and Riesz, 2000; Miyoshi *et al*., 2000; Rosenthal *et al*., 2004) (Figure 6.1).

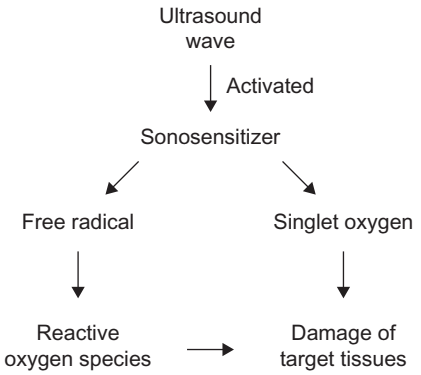

FIGURE 6.1 General principles of sonodynamic therapy.

Compared with other therapeutic modalities, sonodynamic therapy has unique advantages including being safe, cheap, noninvasive, and having fewer side effects. Thus, sonodynamic therapy has extensive potential in clinical treatment of malignant tumors. In order to develop sonodynamic therapy as a novel technique in clinical medicine, we have successfully set up a series of low-intensity ultrasound exposure systems and sonodynamic inactivation of tumor cells has been actively investigated in our laboratory (Wang, P. *et al.*, 2010a,b; Wang, X. *et al.*, 2011a,b, 2012).

A PROMISING SENSITIVE DRUG: CURCUMIN

The sensitizer is a key component affecting the therapeutic efficacy of sonodynamic chemotherapy or sonodynamic therapy. Porphyrin derivatives, such as photofrin, protoporphyrin IX, and hematoporphyrin, are main sensitizers commonly used in SDCT or SDT research (Miyoshi *et al.*, 2000). Subsequent studies also found some photosensitizers of nonporphyrin derivatives have significant sonosensitive activity. Methylene blue could enhance the killing activity of low-intensity ultrasound on ovarian cancer cells and apoptotic induction (Xiang *et al.*, 2011). Low-intensity ultrasound in the presence of pyropheophorbide – a methyl ester – has a strong killing effect and causes mitochondrial damage in liver cancer cells. The *in vitro* and *in vivo* studies show that NPe6 is also a potential sensitizer for sonodynamic tumor treatment (Yumita *et al.*, 2011). With the development of sonodynamic therapy, naturally occurring sensitizers are drawing our research interest because they are often safe with fewer side effects. Traditional Chinese herbs have been used in Chinese medicine for over 1000 years. These herbs contain a variety of important photosensitive drugs, such as psoralen, hypericin, hypocrellin, curcumin, etc. (Dahl *et al.*, 1994; Hadwiger, 1972; Liang *et al.*, 1990; Onoue *et al.*, 2011). Our previous studies showed that LED light-activated hypocrellin, hypericin, or curcumin had remarkable photodynamic inactivation effects on tumor cells (Xu and Leung, 2011; Zeng *et al.*, 2010). Recently, our studies found that hypocrellin B from a traditional Chinese herb *Hypocrella bambuase* (Figure 6.2), which is widely used to treat rheumatoid arthritis and gastric and skin diseases, could enhance

FIGURE 6.2 Traditional Chinese herb *Hypocrella bambuase*.

FIGURE 6.3 Traditional Chinese herb *Curcuma longa*.

low-intensity ultrasound-induced cell death and mitochondrial damage in nasopharyngeal cancer cells, implying that hypocrellin B is a potential natural sonosensitizer (Wang, P. *et al.*, 2010a,b). Our other study also showed that sonodynamic action of hypocrellin B could significantly induce apoptotic cell death of hepatocellular carcinoma cells (Wang, K. *et al.*, 2012). Curcumin is another naturally occurring sensitizer from the traditional Chinese herb *Curcuma longa* (Figure 6.3), which is widely consumed in China and India as a dietary adductive and is used in folk medicine. In our laboratory, curcumin was activated by blue light, and the action of photodynamic therapy with curcumin on tumor cells (Zeng *et al.*, 2010) and bacteria was investigated. More recently, we used our ultrasound exposure system to investigate the combinatory effect of curcumin and low-intensity ultrasound. Our data have shown that ultrasound treatment in the presence of curcumin significantly inactivated nasopharyngeal cancer cells, induced apoptosis, and caused ultrastructural damage and the dysfunction of mitochondria of cancer cells (Wang, X. *et al.*, 2011a,b), demonstrating that curcumin is a promising sensitizer and that curcumin-mediated

sonodynamic chemotherapy might be a potential alternative to treat nasopharyngeal cancer. However, the exact mechanisms need to be clarified. Our recent study observed more mitophagies in nasopharyngeal cancer cells treated by ultrasound sonication in the presence of curcumin, indicating mitophagy induction is an indispensable event in curcumin-mediated sonodynamic chemotherapy on nasopharyngeal cancer cells.

DISCUSSION

Our previous study showed that intracellular reactive oxygen species (ROS) level markedly increased in tumor cells treated by ultrasound sonication in the presence of curcumin (Wang, X. *et al*., 2011a). It is well-known that excessive ROS accumulation in tumor cells is an important cause of cell death. Taking our previous data together, intracellular ROS accumulation leading to mitochondrial morphology and functional damage might be one of the causes of cell death after ultrasound treatment in the presence of curcumin. Cell death usually occurs through apoptosis, necrosis and autophagy. Apoptosis and necrosis of cell death induced by sonodynamic chemotherapy have widely been investigated (Wang, X. *et al*., 2012a; Xiang *et al*., 2011). Different from necrosis and apoptosis, autophagy is a genetically programmed cell death usually accompanied by autophagosome formation with the features of an isolation membrane encapsulating peroxisomes or cytoplasmic components (Li *et al*., 2008; Sy *et al*., 2008; Wang, X. *et al*., 2012b). Autophagy, or "self-eating", has been attracting researchers' attention since Christian de Duve first described it as a lysosome-mediated degradation process in 1963 (Lee *et al*., 2012). Recent studies have shown that autophagy is emerging not only as an essential homeostatic modulator, but also as a quality controller for selectively degrading misfolded proteins and damaged organelles (Lee *et al*., 2012; Li *et al*., 2008; Sy *et al*., 2008). Recently, our study revealed that ultrasound sonication with intensity of $1.35\,W/cm^2$ for 12 sec caused autophagy of nasopharyngeal cancer cells (Wang, P. *et al*., 2011). Ultrasound sonication for 8 sec at an intensity of $0.46\,W/cm^2$ had less effect on cancer cells, but in the presence of curcumin more mitophagies were observed under transmission electron microscopy (TEM) (Wang, X. *et al*., 2012b). These findings demonstrate that ultrasound sonication in the presence of curcumin initiates mitophagy of NPC cells.

Mitophagy is a special kind of autophagy in which damaged mitochondria with dissipated or low mitochondrial membrane potential and activated mitochondrial pore transition are degraded by activated lysosome (Ashrafi and Schwartz, 2013; Hirota *et al*., 2012; Zanchetta *et al*., 2011). Some therapeutic drugs have been shown to induce lethal mitophagy (Setelle *et al*., 2012). Excessive accumulation of intracellular reactive oxygen species (ROS) and mitochondrial damage or membrane potential collapse have been recognized as important inductors of mitophagy (Ashrafi and Schwartz, 2013; Hirota *et al*., 2012; Lee *et al*., 2012; Zanchetta *et al*., 2011). Our previous experimental data from confocal laser scanning microscopy showed that mitochondrial membrane potential significantly decreased in NPC cells treated by ultrasound treatment in the presence of curcumin (Wang, X. *et al*., 2011b), and our TEM results also observed that ultrasound treatment in the presence of curcumin resulted in more significant mitochondrial damage than in the absence of curcumin (Wang, X. *et al*., 2011b). These indicate that ultrasound treatment in the presence of curcumin caused cell death of NPC cells possibly through inducing intracellular ROS increase, damaging

mitochondria and collapsing mitochondrial membrane potential, subsequently initiating lethal mitophagy.

Mitophagy is a complex molecular event involving various upstream and downstream gene molecules. Overproduction of intracellular ROS can initiate autophagy-related genes such as Atg4, p62, and LC3-II, and then activate lysosome to fuse with damaged mitochondria, subsequently inducing mitophagy (Zanchetta *et al*., 2011). Excessive ROS can also open the permeability transition pore of mitochondria and collapse the mitochondrial morphology and functions, directly resulting in mitophagy (Ashrafi and Schwartz, 2013; Hirota *et al*., 2012; Lee *et al*., 2012; Zanchetta *et al*., 2011). Thus, in the combined treatment of ultrasound sonication and curcumin, the accumulating ROS and mitochondrial damage might be important causes of mitophagy in NPC cells after ultrasound sonication in the presence of curcumin. However, which molecules or signaling pathways play key roles in mitophagy induced by ultrasound treatment in the presence of curcumin are still unclear.

Furthermore, growing evidence has shown that mitophagy plays a dual role in determining the fate of cells, i.e., death or survival. On the one hand, mitophagy is essential for homeostatic maintenance. The cell regulates mitochondrial quantity and quality, and maintains accurate repair for surviving through mitophagy to remove the damaged or mutated mitochondria (Kurihara *et al*., 2012; Zanchetta *et al*., 2011). Hughson *et al*. (2012) recognized that cancer cells escaped from the attack of therapeutic drugs and recurred and migrated probably through mitophagical mechanisms. On the other hand, lethal mitophagy can result in cell death and mitophagy induction is regarded as an important strategy for treating malignant tumors (Setelle *et al*., 2012). Therefore, the exact role that mitophagy plays in the mechanisms of sonodynamic chemotherapy of curcumin needs to be clarified in future investigations.

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CHAPTER

7

Role of Nix in the Maturation of Erythroid Cells through Mitochondrial Autophagy

Huanhuan Sun, Lei Wang, Jin Wang, and Min Chen

OUTLINE

Abstract

Selective mitochondrial clearance by autophagy is crucial for maintaining proper cellular function and cellular homeostasis. Reticulocytes, which completely remove their mitochondria during terminal maturation, provide a good physiological model to study the mechanisms of such clearance. Nip-like protein X (Nix, also known as Bnip3L), an atypical BH3-only member of the Bcl-2 family, plays an essential role in

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mitochondrial autophagy occurring during erythroid maturation. $Nix^{-/-}$ reticulocytes show an abnormal retention of mitochondria and a defect in the sequestration of mitochondria by autophagosomes. Nix is not required for autophagosome formation; instead, its role in mitochondrial clearance during erythroid maturation likely involves both dissipation of mitochondrial membrane potential ($\Delta\psi m$) and interaction with autophagosomes. Given that mitochondria are important organelles for energy production and regulation of cell death, elucidating the mechanisms underlying selective mitochondrial autophagy not only will help us to understand the mechanisms for erythroid maturation, but also may provide insights into mitochondrial quality control by autophagy in protection against aging, cancer, and neurodegenerative diseases.

INTRODUCTION

During normal terminal mammalian erythropoiesis, reticulocytes are generated in the bone marrow from orthochromatic erythroblasts by enucleation. Nascent reticulocytes then migrate into the circulation, where they eliminate organelles, including the entire cohort of mitochondria and ribosomes, and mature into erythrocytes (Koury *et al.*, 2002). Autophagy has been shown to play an important role in the degradation of cellular components including organelles, RNA, and proteins in multiple cellular processes (Mizushima *et al.*, 2008). Mitochondrial clearance in reticulocytes has been suggested to occur in an autophagy-related process by early ultrastructural studies (Heynen *et al.*, 1985). However, the molecular mechanisms governing this process have only just begun to be revealed.

Nix (Figure 7.1), an atypical BH3-only member of the Bcl-2 family, is upregulated during terminal erythroid differentiation (Aerbajinai *et al.*, 2003), implicating a potential role for Nix in erythroid differentiation. Nix is localized on the outer mitochondrial membrane and was originally suggested to have pro-apoptotic activity and induce mitochondrial membrane potential loss by binding to Bcl-2 or Bcl-X_L through its BH3 domain (Chen *et al.*, 1999; Imazu *et al.*, 1999). However, cell death induced by overexpression of Nix is delayed and much weaker compared with that induced by other BH3-only proteins such as Bim (Chinnadurai *et al.*, 2008). Furthermore, cell death effect of Nix was not observed in all the cell types and therefore may be dependent on the specific cellular environment and conditions under which Nix is activated. In addition, the transmembrane (TM) domain, but not the BH3 domain, is essential for its apoptotic activity (Chen *et al.*, 1999; Chinnadurai *et al.*, 2008; Imazu *et al.*, 1999). These data suggest that Nix may function differently from classical BH3-only pro-apoptotic proteins. Indeed, using Nix deficient mice, we and others have found that Nix is critical for mitochondrial autophagy during terminal erythroid maturation (Chen *et al.*, 2008; Sandoval *et al.*, 2008; Schweers *et al.*, 2007). Nix is not required for autophagosome formation but is essential for sequestration of mitochondria into autophagosomes. Further studies demonstrate that the role of Nix in mitochondrial clearance during erythroid maturation likely involves both the dissipation of mitochondrial membrane potential ($\Delta\psi m$) and the interaction with autophagosomes (Novak *et al.*, 2010; Sandoval *et al.*, 2008; Schwarten *et al.*, 2009). However, the precise mechanism by which Nix promotes mitochondrial autophagy remains to be defined in detail. Here we review recent advances in our understanding of Nix and its role in mitochondrial autophagy during reticulocyte maturation.

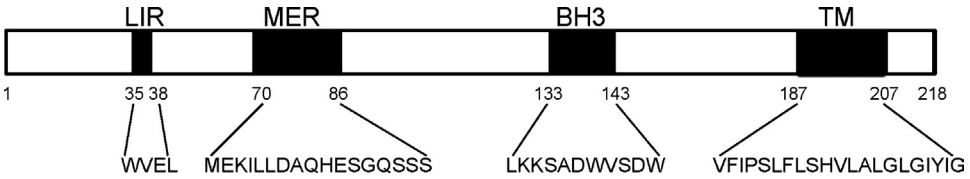

FIGURE 7.1 Domains and functional motifs in murine Nix. Abbreviations: LIR, LC3, interaction region; MER, minimal essential region required for Nix function; BH3, Bcl-2 homology 3; TM, transmembrane domain.

Nix AND MITOCHONDRIAL AUTOPHAGY IN ERYTHROID DIFFERENTIATION

Expression of Nix during Terminal Erythroid Differentiation

Nix was first linked to terminal erythroid maturation in a study comparing the transcriptional profiles of primary human erythroid cells sorted at different stages of differentiation (Aerbajinai *et al.*, 2003). It was reported that Nix expression was upregulated in parallel with Bcl-X_L in human erythroid cells during terminal differentiation. Analysis of the Nix promoter reveals the presence of multiple GC-rich motifs that are bound by transcription factor Sp1 and multiple hypoxia response elements (HRE) that can bind hypoxia-inducible-factor-1 (HIF-1) (Aerbajinai *et al.*, 2003; Galvez *et al.*, 2006). Consistently, Nix expression was upregulated by HIF-1 and p53 under hypoxia in tumor cell lines, as well as in cardiac myocytes treated with agents that activate protein kinase C (Fei *et al.*, 2004; Galvez *et al.*, 2006). Nix was also shown to be activated transcriptionally by Foxo3 in skeletal muscle cells (Mammucari *et al.*, 2007). Analysis of the Human Erythroblast Maturation Database reveals that Foxo3 expression increases upon human erythroid differentiation, which might account for the significant erythroid upregulation of NIX transcripts in bone marrow and reticulocytes, as well as in erythroid cells undergoing terminal differentiation (Aerbajinai *et al.*, 2003; Kang *et al.*, 2012). However, direct activation of Nix expression by Foxo3 remains to be shown in erythroid cells. A recent study by Kang *et al.* (2012) has identified that an additional master regulator of hematopoiesis, GATA-1, can directly activate NIX mRNA expression (Kang *et al.*, 2012). Chip-seq profiling of GATA-1 occupancy revealed that endogenous GATA-1 occupied an intronic site at the NIX locus in primary human erythroblasts (Kang *et al.*, 2012). In addition, GATA-1 occupies the *FOXO3* locus in primary human erythroblasts and can upregulate FOXO3 expression during erythroid differentiation (Kang *et al.*, 2012). Therefore, GATA-1 and Foxo3 may work cooperatively to activate *Nix* gene expression during terminal erythroid differentiation, which may be important for the expression of sufficient Nix protein to promote mitochondrial autophagy in reticulocytes.

Nix Regulates Mitochondrial Autophagy in Reticulocytes

Using $Nix^{-/-}$ mice, we and others have determined the function of Nix during erythroid maturation (Diwan *et al.*, 2007; Sandoval *et al.*, 2008; Schweers *et al.*, 2007). We observed

that $Nix^{-/-}$ red blood cells (RBCs) produce more reactive oxygen species (ROS), display more caspase activation *in vitro*, and undergo faster turnover *in vivo* (Sandoval *et al.*, 2008). Consistently, $Nix^{-/-}$ mice exhibit mild to moderate anemia, with a decreased number of RBCs and compensatory increases of reticulocytes in peripheral blood (Diwan *et al.*, 2007; Sandoval *et al.*, 2008; Schweers *et al.*, 2007). These phenotypes could be explained by the finding that $Nix^{-/-}$ RBCs harbor abnormal mitochondrial retention, which renders them prone to oxidative stress-induced cell death, resulting in shortened lifespan (Sandoval *et al.*, 2008). A significant delay in the removal of the mitochondria was also observed in $Nix^{-/-}$ reticulocytes during *in vitro* maturation (Sandoval *et al.*, 2008), indicating that mitochondrial clearance is defective in the absence of Nix. In contrast, other features of erythroid maturation, including the loss of ribosomes and the downregulation of surface CD71, are normal in the absence of Nix. This suggests that Nix functions specifically for the clearance of mitochondria, but not for the clearance of ribosomes or cellular proteins during erythroid maturation (Sandoval *et al.*, 2008).

Autophagy is implicated in regulating terminal erythroid differentiation (Heynen *et al.*, 1985). Consistently, several autophagy inhibitors, including 3-methyladenine, wortmannin or chloroquine, suppress the clearance of mitochondria in reticulocytes, demonstrating that the mitochondrial removal is an autophagy-dependent process in RBCs (Sandoval *et al.*, 2008). Later studies support this notion showing that the core autophagic proteins, Ulk1 and Atg7, are also required for mitochondrial removal during reticulocyte maturation (Kundu *et al.*, 2008; Mortensen *et al.*, 2010; Zhang *et al.*, 2009). Ultrastructurally, autophagosomal structures were found to be abnormally abundant in $Nix^{-/-}$ RBCs. However, instead of being sequestered into autophagosomes, mitochondria in $Nix^{-/-}$ RBCs tend to cluster outside of autophagosomes (Figure 7.2). These mitochondria either contact with incomplete isolation membrane, or locate on the cytoplasmic face of the autophagosomes (Sandoval *et al.*, 2008; Schweers *et al.*, 2007). LC3 immunofluorescence staining of reticulocytes also indicates that autophagosome formation is not affected by the loss of Nix, but mitochondria and autophagosomes are not co-localized in $Nix^{-/-}$ reticulocytes during *in vitro* maturation (Sandoval *et al.*, 2008). These data support that Nix is not required for the formation of autophagosomes but is likely important for targeting mitochondria to autophagosomes for sequestration and degradation during erythroid maturation.

Nix-Mediated Mitochondrial Clearance Involves Both Atg7-Dependent and Atg7-Independent Autophagy

Two ubiquitin-like conjugation systems, including the Atg12-Atg5 and the Atg8-phosphatidylethanolamine (PE) conjugation pathways, have been shown to be essential for autophagy in yeast (Ohsumi, 2001). Atg7, an E1-like enzyme, is indispensable for the activity of both conjugation pathways, which are required for the growth of autophagosomal membranes. Interestingly, reticulocytes deficient in Atg7 are only partially defective in mitochondrial clearance (Mortensen *et al.*, 2010; Zhang *et al.*, 2009). Moreover, mice with the loss of Atg7 in hematopoietic lineage show a moderate defect in mitochondrial retention in circulating erythrocytes (Mortensen *et al.*, 2010). The reticulocytes deficient in the mammalian homologue of Atg1, Ulk1, which acts upstream of Atg7 as an initiator of autophagy, also display a defect in mitochondrial clearance (Kundu *et al.*, 2008). These data suggest

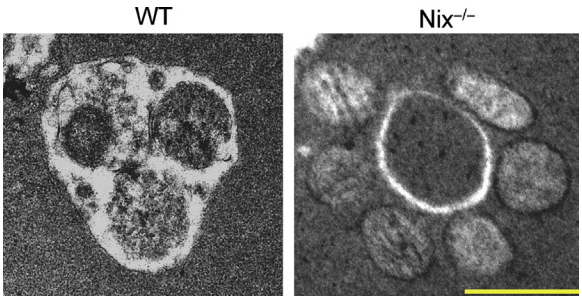

FIGURE 7.2 Analysis of mitochondrial clearance in wild type or Nix-deficient reticulocytes by transmission electron microscopy. Abbreviations: WT, Wild type reticulocyte showing mitochondria sequestered and degraded inside the autophagosome; $Nix^{-/-}$, Nix-deficient reticulocyte showing mitochondria clustered outside the autophagosome (Scale bar: 0.5 μm).

that Nix-mediated mitochondrial clearance may involve an Atg7-dependent autophagy pathway. However, abnormal mitochondrial retention in erythrocytes caused by the loss of Atg7 is less severe than that caused by loss of Ulk1 and Nix, suggesting that Nix may also regulate mitochondrial clearance via an Atg7-independent pathway. Indeed, an alternative autophagy pathway regulated by Ulk1 but independent of Atg5 and Atg7 has been identified (Nishida *et al.*, 2009). This pathway depends on Rab9 for membrane expansion and requires trans-Golgi or late endosomes. It is likely that Nix-mediated mitochondrial clearance in reticulocytes involves both classical and alternative autophagy pathways (Nishida *et al.*, 2009; our unpublished data).

MECHANISMS OF Nix-MEDIATED MITOCHONDRIAL AUTOPHAGY

Nix-Dependent Loss of Mitochondrial Membrane Potential in the Promotion of Mitochondrial Autophagy

Recombinant Nix has been shown to directly induce the loss of mitochondrial membrane potential (Δψm) when incubated with isolated mitochondria (Imazu *et al.*, 1999). Consistently, we found that $Nix^{-/-}$ reticulocytes are defective in the loss of Δψm during *in vitro* maturation (Sandoval *et al.*, 2008). Both an uncoupling agent, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), and a BH3 mimetic, ABT-737, dissipate Δψm and restore the sequestration of mitochondria by autophagosomes in $Nix^{-/-}$ RBCs (Sandoval *et al.*, 2008). Therefore, Nix-dependent loss of Δψm appears to promote the specific recognition and sequestration of mitochondria by autophagosomes. Oligomycin, an F_0F_1-ATPase inhibitor, has no effect on the loss of Δψm or mitochondrial clearance in $Nix^{-/-}$ RBCs, suggesting that inhibiting ATP synthesis alone does not promote mitochondrial autophagy (Sandoval *et al.*, 2008). Several ROS scavengers, including catalase and tempol, also fail to inhibit the loss of depolarized mitochondria in $Nix^{-/-}$ RBCs after FCCP treatment (Sandoval *et al.*, 2008), indicating that FCCP-mediated rescue of mitochondrial autophagy in $Nix^{-/-}$ RBCs does not require ROS. Together, these data suggest that the disruption of Δψm promotes mitochondrial autophagy during reticulocyte maturation.

How Nix induces mitochondrial depolarization is not known. Typical BH3-only proteins have been shown to exert pro-apoptotic activity by activating downstream Bax or Bak to induce mitochondrial dysfunction. However, Bax and Bak double-deficient reticulocytes clear their mitochondria normally (Schweers *et al.*, 2007), suggesting a Bax/Bak-dependent apoptotic mechanism is not required for Nix-mediated mitochondrial autophagy. Chemical inhibitors of the mitochondrial permeability transition pore (MPTP), cyclosporin A and bongkrekic acid, also have no effect on the mitochondrial clearance in wild-type reticulocytes (Schweers *et al.*, 2007), suggesting that the opening of MPTP is not involved in the Nix-mediated mitochondrial autophagy. Bcl-X_L is coexpressed with Nix during terminal erythroid differentiation. However, mitochondrial clearance is intact in BCL-X_L/BAX/BAK triply deficient reticulocytes (Schweers *et al.*, 2007). Thus, Nix-dependent loss of $\Delta\psi m$ and mitochondrial clearance is independent of Bax, Bak, MPTP, and Bcl-X_L.

Nix likely induces the loss of $\Delta\psi m$ through an undefined protein in a mechanism distinct from the one used by traditional pro-apoptotic proteins, and this type of loss of $\Delta\psi m$ should not be severe enough to cause cell death in reticulocytes. Interestingly, a recent study by Nakamura *et al.* (2012) has shown that Mieap, a p53-inducible protein, interacts with Nix and Bnip3 on the mitochondrial outer membrane to cause a dramatic reduction in $\Delta\psi m$. This physical interaction potentially regulates an opening of a pore, which is different from MPTP, on the mitochondrial membrane. Moreover, the formation of this pore causes reduction in $\Delta\psi m$ but does not induce cell death (Nakamura *et al.*, 2012). Mieap has been shown to induce degradation of oxidized mitochondrial proteins and damaged mitochondria via the formation of vacuoles or intramitochondrial lysosome-like organelles (Nakamura *et al.*, 2012). However, whether Mieap is required for mitochondria clearance during reticulocyte maturation has not been studied. How Mieap causes the reduction in $\Delta\psi m$ is also unknown.

As a mitochondrial outer membrane protein, Nix contains only a short C-terminus tail in the mitochondrial intermembrane space. This intra-mitochondrial tail potentially interacts with other signaling proteins in mitochondria to disrupt $\Delta\psi m$ across the inner membrane. Aup1p is a mitochondrial phosphatase that promotes mitochondrial autophagy in yeast (Tal *et al.*, 2007). It remains to be determined whether the mammalian homologue of this mitochondrial phosphatase might mediate the Nix-dependent loss of $\Delta\psi m$ to promote mitochondrial autophagy in erythroid cells.

Mitochondria are highly dynamic organelles that constantly undergo cycles of fusion and fission (Chan, 2012). Recent studies have demonstrated that mitochondrial fission is essential for the removal of mitochondria by autophagy, and that inhibition of fission leads to the disruption of mitochondrial autophagy and accumulation of damaged mitochondria inside the cells (Twig *et al.*, 2008). Fission generates segregated daughter mitochondria with different $\Delta\psi m$, and the mitochondria with low $\Delta\psi m$ are less likely to undergo subsequent fusion and have been shown to be cleared preferentially by autophagy (Twig *et al.*, 2008). It is thus possible that Nix-mediated loss of $\Delta\psi m$ could affect mitochondrial fusion and fission dynamics, thereby regulating mitochondrial autophagy. To that end, overexpression of Bnip3, the closely related Nix homologue, induces mitochondrial fragmentation in fibroblasts (Landes *et al.*, 2010; Quinsay *et al.*, 2010). It will be interesting to examine whether Nix-mediated loss of $\Delta\psi m$ promotes mitochondrial fission required for mitochondrial autophagy.

Nix-induced mitochondrial depolarization could cause changes on the mitochondrial outer membrane, resulting in the recruitment or modification of molecules that then interact

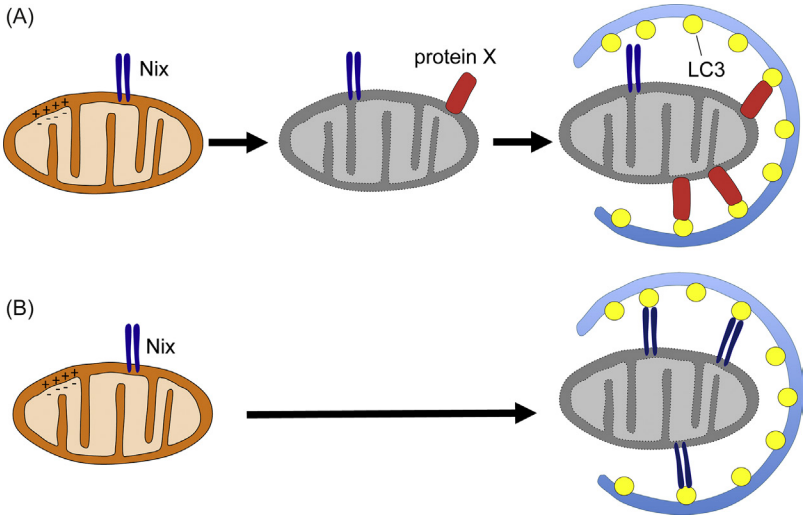

FIGURE 7.3 A model for Nix-dependent mitochondrial autophagy in erythroid differntiation. In differentiating erythroid cells, maturation signals may activate Nix to dissipate $\Delta\psi m$ in mitochondria, leading to the recruitment or modification of protein X on the outer mitochondrial membrane. The interaction between protein X (A) or Nix itself (B) on mitochondria with components of autophagic machinery may lead to the sequestration of the depolarized mitochondria by autophagosomes.

with autophagosomal proteins to promote sequestration of mitochondria (Figure 7.3). The precise mechanisms governing how depolarized mitochondria might be selectively recognized by autophagosomes for sequestration are not completely understood. Recent studies have shown that depolarization in some cell types might cause selective recruitment of Parkin, an ubiquitin E3 ligase mutated in patients with familial Parkinson's disease, to mitochondria through a PINK1-dependent mechanism (Narendra *et al.*, 2008). Parkin recruited to the depolarized mitochondria then mediates the ubiquitination of proteins on the mitochondrial outer membranes, followed by engulfment of mitochondria by autophagosomes for selective clearance (Narendra *et al.*, 2008). Nix has been shown to promote Parkin-dependent mitochondrial autophagy upon mitochondrial depolarization in a hypoxia model (Ding *et al.*, 2010). However, whether Parkin is involved in Nix-dependent mitochondrial autophagy in erythroid cells remains to be determined.

Nix as a Mitochondrial Receptor for Binding to Autophagosome

In addition to the induction of mitochondrial depolarization, some studies suggest that Nix may interact with the autophagosome directly to mediate mitochondrial autophagy. Recently, Atg32, an outer mitochondrial membrane protein, was identified to mediate selective degradation of mitochondria in yeast (Kanki *et al.*, 2009; Okamoto *et al.*, 2009). Atg32 can bind to Atg8 on the isolation membrane via the WXXL-like motif. However, homologues of Atg32 have not been found in mammalian cells. Although Nix bears no sequence homology to Atg32, it does have a candidate Atg8/LC3 binding site containing a W/YxxL/I core motif in the N-terminus (amino acids 35–38 in murine Nix), which resembles the

LC3-interaction region (LIR) identified in several other proteins (Novak *et al.*, 2010; Schwarten *et al.*, 2009). It has been reported by Schwarten *et al.* (2009) that Nix binds directly to GABARAP, an Atg8 homologue, through the WXXL motif. Point mutation of the tryptophan at amino acid 36 to alanine in human Nix abolishes the interaction of a Nix-derived peptide comprising WXXL motif to GABRAP *in vitro*. Further study by Novak *et al.* (2010) showed Nix interacted with LC3 on autophagosomes both *in vivo* and *in vitro*; moreover, a W35A mutation of the WXXL motif in murine Nix (corresponding to W36A in human Nix) abolished the interaction with LC3, partially inhibited mitochondrial autophagy induced by CCCP in mouse embryonic fibroblasts (MEFs), and delayed mitochondrial clearance in reticulocytes during *in vitro* maturation. Therefore, the WXXL motif in Nix may potentially mediate direct binding with LC3 conjugated to the autophagosomes, leading to the recruitment or sequestration of mitochondria into autophagosomes. However, circulating erythrocytes from mice reconstituted with bone marrow cells expressing the Nix mutant carrying the W35A mutation did not display defects in mitochondrial clearance (Novak *et al.*, 2010). This finding suggests that Nix has other mechanisms to interact with autophagic machinery. It may also mediate alternative autophagy pathway for mitochondrial clearance.

Nix Domains Required for Mitochondrial Autophagy

Nix is evolutionarily highly conserved with an atypical BH3 domain, a transmembrane domain (TM), and a short C-terminal tail (Figure 7.1). Its BH3 domain differs from the [L/V/M^1-X^2-X^3-X^4-G^5-D^6-D/E^7-F^8-E^9-R^{10}] canonical BH3 domain sequence in two conserved tryptophan residues at amino acid 139 and 143 in murine Nix, which may account for its inconsistent and much weaker death-inducing functions in comparison with other typical BH3-only proteins (Chinnadurai *et al.*, 2008). The BH3 domain of Nix could induce autophagy under hypoxic conditions (Bellot *et al.*, 2009). However, this domain does not seem to be required for autophagy induction during reticulocyte maturation, since formation of autophagosomes is normal in the absence of Nix (Sandoval *et al.*, 2008; Schweers *et al.*, 2007).

The TM domain and the C-terminal region are required for dimer formation and mitochondrial localization of Nix (Chen *et al.*, 1999; Imazu *et al.*, 1999). Deletion of the TM domain and the C-terminal tail disrupts the interaction between Nix and Bcl-2 completely (Imazu *et al.*, 1999) and also causes mitochondrial retention in circulating erythrocytes similar to that caused by the loss of Nix (Zhang *et al.*, 2012). These data suggest that Nix homodimerization or mitochondrial localization is important for mitochondrial autophagy.

By generating a series of deletion mutants of Nix, Zhang *et al.* found that a minimal essential region (MER) in Nix, encompassing amino acids 70–86, is required for mitochondrial clearance in reticulocytes (Zhang *et al.*, 2012). They further identified that a single amino acid mutation of L74A in murine Nix could disrupt Nix function in mitochondrial clearance. Interestingly, when the N-terminal LC3 interacting region LIR or the BH3 domain was deleted in Nix, rescue of mitochondrial clearance in Nix deficient reticulocytes by those mutants could still be observed. These data suggest that the LIR or the BH3 domain is not essential for Nix-mediated mitochondrial autophagy. However, it remains possible that the LIR or BH3 domain may help to make mitochondrial autophagy more efficient.

The MER region is predicted to have an α-helical secondary structure with a critical hydrophobic leucine residue and several flanking charged amino acids contributing to

Nix function (Zhang *et al.*, 2012). This motif may mediate the interaction with other proteins required for mitochondrial autophagy through the predicted α-helix. The MER region does not bind to LC3; however, it may interact with other proteins on the autophagosomes. Alternatively, it may interact with components required for alternative autophagy.

Recent studies have shown that the small GTPase Rheb interacts with Nix and regulates mitochondrial turnover through autophagy in response to mitochondrial energetic status (Melser *et al.*, 2013). Rheb is recruited to the outer mitochondrial membrane upon increased mitochondrial oxidative phosphorylation activity and may facilitate the interaction between Nix and LC3. Whether Rheb is involved in Nix-dependent mitochondrial autophagy during reticulocyte maturation remains to be determined. It will be interesting to examine whether Nix interacts with Rheb through the MER region.

CONCLUSIONS

Studies by our laboratory and others have demonstrated that Nix is required for mitochondrial autophagy during reticulocyte maturation. Nix could induce mitochondrial depolarization, or function as a mitochondrial receptor, by binding to LC3/Atg8 homologue on the autophagosomes, leading to specific recognition and sequestration of mitochondria by autophagosomes during erythroid maturation (Figure 7.3). However, several questions remain to be answered regarding how Nix accomplishes its functions. How is the function of Nix regulated with regard to its role in the induction of mitochondrial depolarization and its interactions with the autophagic machinery? Are post-transcriptional or post-translational mechanisms involved? How is the alternative autophagy pathway related to Nix-mediated mitochondrial autophagy? What are the identities of Nix-interacting proteins required for Nix to achieve its functions? Recent delineation of the minimal region in Nix required for mitochondrial autophagy will undoubtedly facilitate the identification of such proteins. The retention of damaged mitochondria is harmful to the cells due to cytotoxicity and cell death induced by the elevated ROS generated from oxidative metabolism. Mitochondrial clearance is important not only for erythroid maturation, but also for regulating mitochondrial numbers and quality control in other cell types. Therefore, studying molecular mechanisms underlying mitochondrial autophagy in reticulocytes may shed light on the role of mitochondrial autophagy in the development and function of other cell types, as well as in various disease processes including autoimmunity, cancer, and cardiovascular diseases.

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CHAPTER

8

Role of the Antioxidant Melatonin in Regulating Autophagy and Mitophagy

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OUTLINE

Abstract

Autophagy is an evolutionarily conserved and highly regulated lysosomal related process that degrades oxidatively damaged, aberrant macromolecules and organelles for the purpose of maintaining homeostasis during stress. Several types of autophagy, including pexophagy, ER-phagy, or ribophagy, have been described, based on which organelles are targeted for specific autophagic degradation. Among them, mitophagy plays a crucial role in the well-being of cells, since it is the major degradative pathway in mitochondrial turnover. Although autophagy is emerging as an important mediator of pathological responses and engages in cross-talk with ROS (reactive oxygen species) and RNS (reactive nitrogen species) in both cell signaling and protein damage, the role of antioxidants in the regulation of these processes has been sparingly investigated. Melatonin, which is a ubiquitously acting antioxidant with protective actions against oxidative stress, also exhibits beneficial effects against mitochondrial dysfunction, suggesting a relation to mitophagy. This chapter focuses on data and the most recent advances related to the role of melatonin in health and disease, on autophagy activation in general, and on mitophagy in particular.

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INTRODUCTION

Autophagy is an evolutionarily conserved catabolic process in which eukaryotic cells remove cytosolic components such as damaged proteins and organelles. This process not only eliminates oxidatively damaged, aberrant macromolecules and organelles for the purpose of maintaining homeostasis during stress but also allows the cells to avoid starvation by recycling the damaged components

Upon autophagy induction, a membrane structure termed the phagophore is formed and gradually expanded to form a double-membrane autophagosome that engulfs a portion of the cytosol or specific molecular cargos and delivers them to the vacuole/lysosome for degradation. The mechanism of autophagosome formation provides autophagy with the capacity to sequester essentially any cellular component and deliver it into the vacuole for degradation.

Autophagy, which has been studied at molecular level in yeasts and mammals, is controlled by the action of more than 30 autophagy-related proteins (collectively termed Atgs). mTOR Complex 1 (mTORC1), which is formed by the serine/threonine kinase mammalian target of rapamycin (mTOR), the regulatory-associated protein of mTOR (RAPTOR) and the mammalian LST8/G-protein β-subunit like protein (mLST8/GβL), as well as the Unc-51-like kinase (mULK) complex consisting of ULK1/2, mAtg13, FIP200, and Atg101, are central components of the core machinery involved in autophagosome induction. Thus, in low energy levels, the AMP-activated protein kinase (AMPK) is activated and phosphorylates RAPTOR through the tuberous sclerosis complex (TSC) tumor suppressors (TSC1 and TSC2) and the Ras-related small G protein Rheb leading to inactivation of mTORC1. Inactivated mTORC1 dissociates from the ULK kinase complex, which is free to phosphorylate other components of the ULK kinase complex, such as mAtg13 and FIP200, leading to autophagy induction (Mizushima and Levine, 2010). A recent report has suggested that ULK1 can also be directly phosphorylated by AMPK (Egan *et al*., 2011).

The subsequent development of autophagosomes is dependent on a class III phosphatidylinositol-3 kinase (PI3K), also known as Vps34, complex. The complex containing Beclin 1–UVRAG (UV radiation resistance associated gene) and Beclin 1–Ambra1 activates autophagy, while the Beclin 1–Rubicon complex negatively regulates class III PI3K activity and inhibits autophagy. The class III PI3K complex localizes to the isolation membranes and recruits further Atgs including Atg3, Atg5, Atg7, and microtubule-associated protein light chain 3 (LC3), to allow for elongation of the isolation membranes and completion of the autophagosomes. Mediated by the lysosomal membrane protein LAMP-2 and the small GTPase Rab7, autophagosomes fuse with lysosomes, leading to the breakdown of proteins and organelles by lysosomal enzymes (Yang and Klionsky, 2010).

Whether autophagy promotes or prevents cell death remains controversial. Thus, an excessive and dysregulated autophagy is often a prominent feature of programmed cell death, with autophagosomes spreading throughout the cell and swallowing cytoplasmic components resulting in progressive loss of electron density until the cell dies. Descriptions of autophagic cell death consistently recount degradation of cytoplasmic components preceding nuclear collapse. If autophagy removed damaged organelles that would otherwise activate programmed cell death as apoptosis, then autophagy would be protective.

Another controversial issue is whether autophagy is selective or nonselective. When autophagy results in cell death, it is obviously not a selective process. In situations where

autophagy develops as a survival procedure, however, controversy still persists, since the fact that cytosolic enzymes with different half-lives are sequestered at similar rates during autophagy, and autophagosomes often contain a variety of different cytoplasmic elements, e.g., cytosolic proteins and organelles such as endoplasmic reticulum, peroxisomes, and mitochondria, support the assumption that autophagy is a nonspecific form of lysosomal degradation.

Many recent findings have revealed that there are specific types of autophagy in which particular proteins or organelles are delivered to the autophagosome/lysosome for degradation. These types of autophagy include selective degradation of peroxisomes (pexophagy), endoplasmic reticulum (reticulophagy), mitochondria (mitophagy), ribosomes (ribophagy), microorganisms (xenophagy), and portions of the nucleus (nucleophagy) during which parts of the nucleus are specifically degraded by an autophagic process. Of these autophagy types, mitophagy plays a crucial role in the well-being of cells, since it is the primary mechanism for the elimination of these essential organelles and the major degradative pathway in mitochondrial turnover (Kanki, 2010). In yeast, Atg32, a mitochondrial outer membrane protein, functions as a receptor protein to deliver mitochondria to the vacuole. Atg32 interacts with Atg11, an adaptor protein, and Atg8, through an Atg8-binding sequence (WXXL motif or a similar sequence), and is then recruited and imported into the vacuole along with mitochondria (Kondo-Okamoto *et al*., 2012).

Although a mammalian homologue of the yeast mitophagy receptor Atg32 has not been clearly identified, recent papers suggest that BCL2/adenovirus E1B 19 kDa-interacting protein 3 (BNIP3) and BNIP3-like (BNIP3L), also known as NIX, which are two mitochondrial outer membrane proteins required for mitochondrial clearance during erythrocyte maturation (Kanki, 2010), may be mitochondrial receptors for mitophagy in mammalian cells. To support this fact, recent studies have shown that both BNIP3 and NIX bind to Atg8 mammalian homologues, LC3 and gamma-aminobutyric acid receptor-associated protein (GABARAP); these are ubiquitin-like modifiers that are required for the growth of autophagosomal membranes (Hanna *et al*., 2012). Furthermore, BNIP3 competes with Beclin 1, a highly conserved protein that is required for the initiation of autophagy, for binding to Bcl-2, an anti-apoptotic protein, and thereby increases the levels of free Beclin 1, which triggers autophagy (Zhang *et al*., 2008).

Although the elimination of damaged or malfunctioning mitochondria is essential for maintaining cellular homeostasis (Kanki, 2010), mitochondrial energy supply has to be maintained for essentially all cellular activities to occur. Mitochondria are the site of oxidative phosphorylation, which generates ATP coupled to electron transfer from respiratory substrates to oxygen by a series of oxidation-reduction reactions that pump protons across the mitochondrial inner membrane from the matrix space. In mitochondria and submitochondrial particles, respiration produces reactive oxygen species (ROS) including superoxide anion ($O_2 \cdot^-$) and hydrogen peroxide (H_2O_2); this occurs especially if respiration is inhibited or otherwise disordered. This documents that mitochondria are the major source of ROS production and, at the same time, organelles particularly prone to ROS damage. It is generally accepted that ROS induce autophagy and that autophagy, in turn, serves to reduce the accumulation of oxidatively damaged molecules and organelles (Dutta *et al*., 2013).

Excessive generation of ROS and reactive nitrogen species (RNS) leads to oxidative stress, a condition in which cellular constituents including proteins, DNA, and lipids are oxidized

and damaged. To protect themselves against free radical-mediated damage, cells have developed an antioxidant defense system which includes enzymatic and nonenzymatic mechanisms. Free radical generation and a functionally efficient antioxidant defense system must be in equilibrium to avoid cellular damage caused by radicals and their derivatives. Enzymes involved in the elimination of free radicals include the superoxide dismutases (SOD), catalase (CAT), and glutathione peroxidase (GPx). In addition to the enzymatic antioxidant system, organisms possess nonenzymatic free radical scavengers, which directly remove toxic reactants because of their electron donoring ability. The best known nonenzymatic antioxidants are vitamin E (α-tocopherol), vitamin C (ascorbate), glutathione (GSH), β-carotene and, as recently described, melatonin (Galano *et al*., 2011).

Melatonin, which was originally identified as a product of the mammal pineal gland, is a powerful and effective free radical scavenger, which provides protection against oxidative damage of cell components. The high efficiency of melatonin as an antioxidant depends on several characteristics: it is highly lipophilic and weakly hydrophilic, and can cross morphophysiological barriers, including the placenta and the blood-brain barrier, and it easily enters cells. It is available to all tissues and cells, and distributes in all subcellular compartments, being especially high in the nucleus, cell membrane, and mitochondria (Venegas *et al*., 2012). This means that melatonin is available at the sites in which free radicals are being maximally generated, thereby decreasing the potential molecular damage. Furthermore, melatonin stimulates several antioxidative enzymes including GPx, CAT, and SOD, thus potentiating its antioxidant properties.

Although the regulatory effects of melatonin on apoptotic cell death based on its antioxidant properties have been extensively investigated both *in vivo* and *in vitro* (Leja-Szpak *et al*., 2010), few reports have studied its role in ROS/RNS-mediated autophagy. Furthermore, the relationship between melatonin and mitophagy remains unclear, even taking into account melatonin's critical role in maintaining mitochondrial homeostasis, which is based on three main capabilities: (1) the mitochondrion is the organelle with the highest ROS/RNS production in the cell and melatonin is a powerful scavenger of these reactive species; (2) mitochondria depend on glutathione (GSH) uptake from the cytosol and melatonin improves the GSH redox cycling and increases GSH content by stimulating its synthesis in the cytosol; (3) melatonin exerts important anti-apoptotic effects in normal cells and the majority of these signals originate from the mitochondria.

Herein, we summarize and interpret recent advances in our understanding of melatonin's potential role in the regulation of autophagy, especially mitophagy, in health and disease. The data discussed illustrate the ubiquitous actions of melatonin in multiple cell types.

AGING

The free radical theory of aging proposes that aging results, at least in part, from damage to cellular components by ROS. Thus, cellular proteins, lipids, and nucleic acids sustain damage that can lead to functional deficits in tissues and, ultimately, to organ and organismal death. Levels of oxidative damage correlate with chronological age and extent of functional decline. Since oxidative stress is believed to be a major component of human aging, autophagy, which serves to reduce oxidative damage, has been implicated in the

degenerative processes of aging and, thus, it is accepted that ROS-induced autophagy is increased during aging. To study the mechanisms related to the process of aging, the senescence-accelerated prone mouse 8 (SAMP8) was used as an animal model. ATP production and respiration in brain mitochondria from old SAMP8 mice are lower than in mitochondria from senescence-accelerated resistant mice 1 (SAMR1) (Carretero *et al*., 2009). Persistent or extensive damage was also observed in brain of SAMP8 animals, which increased protein aggregation, a phenomenon that may represent an age-related diminishment of proteolytic cleavage and, with it, a reduction in autophagic processes. Related to this, the measurement of the activities of major lysosomal proteases, cathepsin D and B, which are abundantly expressed in the brain, shows a decreased activity and expression of cathepsin D, together with a rise of cathepsin B activity with increased age. Alterations in lysosomal proteolytic activity would likely influence the elimination of damaged/aged structures (Vega-Naredo and Coto-Montes, 2009). In support of this conclusion, Caballero *et al*. (2009) have studied apoptosis and autophagy in brain of 10-month-old SAMP8 mice and they observed a lack of apoptotic and autophagic processes in these animals. High Beclin 1 levels were observed in SAM brain, together with a lack of LC3-II proteins, which may be associated with alterations in the autophagosome formation in old organisms. A reduction in autophagy with age has been observed in most tissues and organisms and malfunction of this process has been proposed as a feature of the aged cell.

This fact is particularly observed during aging of the immune system, where age-related changes of immune function increase susceptibility to infectious diseases, autoimmunity, and cancer. In immune cells, autophagy, which is referred to as immunophagy, has an essential role in the adaptive immunity for antigen processing, a consequence of antigen cleavage by lysosomal proteases such as cathepsins. Spleens from aged SAMP8 mice (10 months of age) exhibit immunosenescence associated with a redox imbalance and a decline in antioxidant defense and protease activity (Caballero *et al*., 2009). Pathways involved in autophagy are upregulated in old SAMP8 mice as an adaptive response to oxidative stress in the spleen from both males and females, with high LC3-II detection and increased Beclin 1 levels. Previous reports have proposed that increased autophagy, which removes oxidatively damaged organelles such as mitochondria and mutilated proteins, provides a second level of defense, when antioxidant activities are compromised. This response provides a selective advantage in conferring resistance to excessive oxidative stress.

Because mitochondria are a major site of ROS generation, mitochondrial dysfunction has been correlated with aging (Sena and Chandel, 2012). ROS generation in mitochondria leads to mitochondrial protein and mtDNA damage because of the fact that mtDNA is more sensitive and susceptible to oxidative damage due to lack of histones and a lower efficiency of mtDNA repair mechanisms compared to nuclear DNA. Thus, mitichondrial protein damage and mutations of mtDNA accumulate during aging. In postmitotic cells of aged organisms, morphological abnormalities of mitochondria are often observed including swelling, loss of cristae, and destruction of their inner membranes (Caballero *et al*., 2008).

Caloric restriction is known to promote longevity in species from yeast to mammals. Deletion of the yeast outer mitochondrial membrane protein, Uth1, which is essential for the specific autophagic elimination of mitochondria upon nitrogen starvation, results in a selective defect in mitophagy and decreased lifespan upon nutrient deprivation. Given that caloric restriction induces autophagy, increased longevity may in part be a result of

enhanced elimination of dysfunctional mitochondria. Other mechanisms involving lifespan and mitophagy have been described in *C. elegans*. Thus, worms that carry the loss-of-function mutation in the insulin-like signaling pathway (*daf-2*) display extended lifespans and induction of autophagy (Yen and Klionsky, 2008).

The fact that endogenous melatonin levels exhibit a profound reduction in aged animals including humans suggests a relationship between this indoleamine and aging. In fact, treatment with melatonin has often been used to reduce age-related molecular damage. Clearly, administration of this indoleamine causes a reduction of oxidative stress-related impairments, with a rise in antioxidant enzyme activities, a decrease in protease activities, and modifications of apoptotic markers. However, changes in autophagy in the brain of aging animals are virtually uninvestigated (Caballero *et al*., 2009). In the case of the immune system, melatonin and autophagy may work synergetically in a pro-survival function, reducing oxidative stress and delaying immunosenescence. Other experiments designed to establish melatonin's role during immunosuppression due to cyclosporine treatment, but not during aging, produced similar results. Thus, autophagy was increased during oxidative stress mediated by cyclosporine while it was suppressed by treatment with melatonin, which inhibits LC3-II expression (Yoo and Jeung, 2010). Obviously, additional experiments that examine the effect of melatonin on autophagy during aging are necessary to clarify the role, if any, of this important antioxidant in processes that so markedly impact age-related degeneration.

NEURODEGENERATIVE DISEASES

Neuronal processes, such as axonal transport of macromolecules and organelles, maintenance of membrane potential, loading and releasing neurotransmitters, and buffering cytosolic calcium dictate a high-energy demand. Since most neuronal ATP is generated through oxidative phosphorylation, neuronal survival and activity are critically dependent on integrity and functionality of mitochondrias that are abundant in neuronal cells. Hence, mitochondrial damage and deregulation of mitophagy has been implicated in the onset and progression of several age-associated neurodegenerative diseases, such as Parkinson's (Schapira, 2011), Alzheimer's, and Huntington disease (Batlevi and La Spada, 2011). Removal of aberrant mitochondria has been shown to play a protective role in age-related neurodegenerative disorders (Mammucari and Rizzuto, 2010).

Mutations and/or deletions of mtDNA commonly appear and accumulate during aging in mitochondria of neurons of the substantia nigra; this is accompanied by the accumulation of mitochondrial damage and loss of dopaminergic neurons at the substantia nigra, a region important for motor control and coordination. Loss of dopaminergic neurons in the substantia nigra is the major feature of Parkinson's disease (PD). In fact, mtDNA mutations are more frequent in patients with PD compared to age-matched individuals in the population (Bender *et al*., 2006). The mitochondrial phosphatase and tensin homologue (PTEN)-induced kinase 1 (PINK1) phosphorylates and recruits Parkin, a cytosolic E3 ubiquitin ligase, to the mitochondria. Ubiquitination of outer membrane mitochondrial proteins including Mfn1/2, VDAC, and MIRO1/2 by Parkin induces the recruitment of the autophagic machinery. Loss-of-function mutations in *PINK1* and/or *PARK2* genes have been linked to the early onset of hereditary forms of PD. Thus, PINK1 appears to play an important role in the maintenance

of mitochondrial networks which, together with Parkin-induced mitophagy, may serve to reduce toxicity associated with dysfunctional mitochondria in PD (Matsuda *et al.*, 2010; Vives-Bauza *et al.*, 2010).

The potential role of melatonin in autophagic regulation in PD has been recently studied in the SK-N-SH dopaminergic cell line treated with methamphetamine (METH), a neurotoxin which causes neurological symptoms similar to those of PD (Nopparat *et al.*, 2010). In the SK-N-SH dopaminergic cell line, METH inhibits the phosphorylation of mTOR and induces the expression of LC3-II in a dose-dependent manner. Moreover, METH also inhibits the dissociation of the Bcl-2/Beclin 1 complex and its upstream pathway that promotes cell death. In cells exposed to METH, melatonin enhances mTOR activity and reduces the formation of LC3-II (Kongsuphol *et al.*, 2009), as well as protects cells from autophagic cell death by blocking of Bcl-2/Beclin 1 dissociation, since it inactivates c-Jun N-terminal kinase 1 (JNK 1), which is upstream of Bcl-2 phosphorylation (Nopparat *et al.*, 2010).

Alzheimer's disease (AD) is the most common age-associated neurodegenerative disorder, characterized by cognitive dysfunction and loss of memory. This disease is accompanied by accumulation of distinctive intracellular neurofibrillary tangles and extracellular amyloid plaques composed of beta-amyloid derived from amyloid precursor protein (APP), which induce neuronal cell death in cerebral cortex. Taking into account that the accumulation of beta-amyloid fragments in mitochondria as well as abnormalities in mitochondrial structure have been observed in afflicted individuals, mitochondrial damage has been implicated in the development and progression of AD. In addition, the presence of autophagic vacuoles in neurons of Alzheimer's disease patients further implicates cytoplasmic and organelle-specific degradation in disease progression. In this context, mitophagy may have a pivotal role in ameliorating or defending against the development of AD through elimination of defective mitochondria which carry cytotoxic beta-amyloid fragments. Studies of melatonin effects on autophagic processes in AD brains should consider taking into account autophagic observations in the brains of PD patients as described above and a growing body of evidence related to beneficial effects of melatonin in AD (Dragicevic *et al.*, 2011; Rosales-Corral *et al.*, 2012).

CANCER

Autophagy plays a dual role in tumorigenesis. The fact that autophagy promotes cell death supports its role as a tumor suppressor and its use as a therapeutic strategy. Furthermore, autophagy has been shown to induce cell senescence, which impedes cancer progression (Young *et al.*, 2009). Autophagy can also prevent cell death and act as an oncogenic mechanism. Since autophagy upregulation provides tumor cells with several survival advantages (Lee *et al.*, 2012), inhibition of autophagy has been used as a tumor cell strategy. Experimental evidence suggests that autophagy is activated as a prosurvival mechanism against cytotoxic agents by helping to clear the drug-induced damage resulting in cancer cells resistant to chemotherapy (Yang *et al.*, 2011). Several colorectal cancer cells or cell lines treated with pharmacological inhibitors of autophagy showed increased sensitivity to cyclooxygenase inhibition, TRAIL-induced cell death, amino-acid deprivation, sulforaphane-induced apoptosis and 5-fluorouracil chemotherapy.

The highly proliferative nature of solid tumors leads to the rapid consumption of nutrients and oxygen causing tumor cells to easily suffer from nutrient deprivation and hypoxia. In this situation, hypoxia-inducible-factor (HIF-1), which is a transcription factor involved in the cellular response to hypoxia, is stimulated, and activates various target genes that control cell metabolism and promote cell survival. A positive role for HIF1 in tumor cell survival is also attributed to its activation by deregulation of mTOR (a regulator of cell proliferation that inhibits autophagy), which results in increased HIF-1α translation. Moreover, HIF-2α is thought to regulate mitochondrial superoxide dismutase (SOD2) resulting in a reduction of ROS. Hypoxia-induced activation of HIF-2α (but not HIF-1α) is facilitated by sirtuin-1, which induces autophagy via BNIP3-induction (Semenza, 2010).

Another pathway, which supports the oncostatic role for autophagy, involves ataxia telangiectasia mutated (ATM), a cellular damage sensor that coordinates the cell cycle with damage-response checkpoints and DNA repair to preserve genomic integrity. In response to H_2O_2, ATM activates tumor suppressor molecules, including TSC2; this results in inhibition of mTOR and induction of autophagy in a p53-independent manner. Moreover, the autophagy activator (and mTOR inhibitor), rapamycin, reduces lymphogenesis of ATM-deficient mice, thus suggesting an antitumor role for autophagy in this system (Alexander *et al.*, 2010).

Although only a few studies have examined the role of mitophagy in tumorigenesis, mitophagy-related genes *BNIP3* and *NIX* in tumors have been investigated. Although activation by hypoxia of these genes in normal cells often results in cell death rather than survival, in tumor cells activation of BNIP3-dependent autophagy reduces mitochondrial mass and ROS formation, thus enhancing tumor cell survival and promoting cancer. In human carcinoma cell lines, hypoxia induces BNIP3 expression in perinecrotic areas of several epithelial cell carcinomas through the HIF-1α pathway. HIF-1α expression was suppressed by von Hippel-Lindau protein in normoxic cells. Hypoxia in tumors is a negative prognosis indicator; accordingly, deregulation of BNIP3 expression is associated with an aggressive disease (Burton and Gibson, 2009).

Regulation of NIX by hypoxia in tumor cell lines has also been reported. In U2OS osteosarcoma cells, NIX is regulated by hypoxia involving HIF-1α-dependent recruitment of CBP [cAMP response element-binding protein (CREB)-binding protein] to the *NIX* gene, followed by recruitment of p53. Experimental downregulation of NIX increased the growth of these cells in a tumor transplant model, suggesting that NIX may inhibit tumor growth under hypoxic conditions. Thus, BNIP3 and NIX contribute to hypoxia-induced cell death and tumor suppression (Zhang and Ney, 2009).

Whether autophagy promotes or prevents tumor cell death remains controversial. Both processes are possible with the difference being linked to the cellular state. If autophagy promotes cell survival, it will occur; however, if insufficient autophagic effort is expended in degrading damaged macromolecules or organelles, the cell increases the autophagic response and the cell undergoes programmed cell death type II, triggering apoptosis (Vega-Naredo and Coto-Montes, 2009). The regulation of the different responses may relate to the degree of ROS generation and, thus, antioxidants which scavenge free radicals would be expected to modulate both autophagic processes. This intricate relationship forces a cautious evaluation of the published findings, since apparently similar circumstances can have very different outcomes.

The ability of melatonin to inhibit the growth of several tumor cell lines has been repeatedly observed. Mechanisms involved in its anticarcinogenic effect are related to melatonin's antioxidant actions, the modulation of the endocrine system, its ability to suppress the uptake of growth factors by cancer cells, its immunoenhancing properties, and its direct effect on cancer cell proliferation (Sánchez-Hidalgo *et al.*, 2012).

Although melatonin's regulatory role in autophagy regulation in cancer remains poorly understood, a recent review discussed the role of melatonin in colon cancer suggesting that the anti-oncogenic activity of melatonin may be in part due to its anti-autophagic properties (Motilva *et al.*, 2011). It can be assumed that the anti-autophagic action of melatonin relates to the capability of this indoleamine to reduce ROS, since in these cases the autophagic processes involve the removal of damaged mitochondria; thus, the anti-autophagic properties described are mainly mitophagy.

In H4IIE hepatoma cells, hydrogen peroxide (H_2O_2) stimulated the mitogen-activated protein kinases MAPK and mTOR signaling pathways through the activation of Ras. Treatment with melatonin dramatically attenuated the phosphorylation of downstream targets including mTOR and mTOR substrates by preventing Ras activation (Kimball *et al.*, 2008) Thus, melatonin increases autophagy by mTOR-downregulation and, also, these changes may relate to alterations in ROS levels due to the free radical scavenging properties of melatonin and its metabolites (Galano *et al.*, 2013).

A recent study has reported that melatonin induces protective autophagy that prevents mouse hepatoma H22 cells from undergoing apoptosis. Melatonin triggers an autophagic process by enhancing Beclin 1 expression and inducing a conversion of LC3-I to LC3-II, the protein associated with the autophagosome membrane, in hepatoma H22 tumor-bearing mice. Moreover, melatonin inhibits the phosphorylation of the mTOR and Akt. The authors suggest that a combination of melatonin with an autophagy inhibitor might be a useful therapeutic strategy for hepatocellular carcinoma (Liu *et al.*, 2012).

CONCLUSIONS AND PERSPECTIVES

Melatonin exhibits opposite roles in regulating apoptosis (programmed cell death type I); thus it increases or reduces this process, in normal and cancer cells, respectively. These contrasting findings can be explained by melatonin's role in modulating oxidative stress by different pathways (direct and indirect), which can induce both cellular actions. An analogous relationship between melatonin and another type of programmed cell death (type II or autophagy) is suggested, since melatonin and autophagy are intimately related to the levels of oxidative stress. In fact, melatonin induces or inhibits autophagy based on cellular necessities and oxidative stress levels. The dual capabilities of autophagy (inducing survival or promoting death depending on cellular stress) supports the need for more careful studies related to the regulatory role of melatonin in the process of autophagy.

Finally, the role of melatonin in mitochondrial physiology, maintaining and/or increasing mitochondrial number and size after its chronic administration while ameliorating mitochondrial dysfunction, suggests a possible regulation of mitophagy by melatonin with potentially important medical implications.

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CHAPTER

9

Ubiquitin Ligase-Assisted Selective Autophagy of Mitochondria: Determining Its Biological Significance Using *Drosophila* Models

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OUTLINE

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Abstract

Mitochondria have important cellular roles in energy supply and the synthesis of fatty acids and lipids, as well as in the cell signaling involved in cell death and immune responses, and the accumulation of dysfunctional mitochondria has deleterious consequences for cells. The selective autophagy of mitochondria, called mitophagy, is known to occur physiologically during the maturation of reticulocytes and during the removal of sperm mitochondria from egg cells upon fertilization. Recent genetic studies of Parkinson's disease (PD) have suggested that mitophagy is also important for mitochondrial maintenance and the elimination of damaged mitochondria during steady-state conditions. Here we review recent advances in the understanding of mitophagy as a form of mitochondrial quality control based on *in vivo Drosophila* models. We then discuss the biological and pathological significance of mitophagy and the potential contributions of *Drosophila* models to the understanding of human diseases caused by defects in mitophagy.

INTRODUCTION

Mitochondria play important roles in cellular activity; however, the accumulation of damaged mitochondria can cause oxidative stress and energy supply failure that eventually lead to cell death. Recent studies on familial Parkinson's disease (PD) have led to the development of a new concept of mitochondrial maintenance in cells that is mediated by a selective autophagy mechanism called mitophagy. Mutations in the *Parkin* gene have been identified in young-onset familial PD cases (Kitada *et al*., 1998), and the gene product Parkin has been characterized as a ubiquitin ligase (Imai *et al*., 2000; Shimura *et al*., 2000; Zhang *et al*., 2000). Although Parkin is mainly localized in the cytoplasm of cultured cells, severe mitochondrial degeneration in the muscle tissues and spermatogenic cells has been reported in Parkin-deficient *Drosophila* (Greene *et al*., 2003). Similar phenotypes have been reported in another *Drosophila* model of young-onset familial PD that involves the gene *PTEN-induced putative kinase 1* (*PINK1*). Additionally, the transient expression of Parkin suppresses the phenotype of PINK1-deficient flies; however, the overexpression of PINK1 does not rescue the *Parkin*-phenotypes, strongly suggesting that PINK1 is an upstream regulator of Parkin (Clark *et al*., 2006). PINK1 is an unstable, mitochondrially located protein kinase (Matsuda *et al*., 2010; Narendra *et al*., 2010; Vives-bauza *et al*., 2010). The loss of the mitochondrial membrane potential induces the mitochondrial localization of Parkin (Narendra *et al*., 2008), and this process is dependent on PINK1 activity. Upon mitochondrial membrane depolarization, PINK1 is stabilized and accumulates on the outer membrane of the damaged and depolarized mitochondria. Accumulated PINK1 is activated via autophosphorylation and recruits Parkin to these abnormal mitochondria. During this event, Parkin is phosphorylated by active PINK1, and mitochondrially localized Parkin is activated and ubiquitinates mitochondrial proteins. This poly-ubiquitination reaction is thought to recruit the autophagy machinery. Although there is no direct evidence of the occurrence of mitophagy *in vivo*, accumulating evidence from studies of *Drosophila* strongly suggests that Parkin regulates mitochondrial quality control, including mitophagy, in cooperation with PINK1. Alternatively, PINK1 might function to regulate mitochondrial activity independent of Parkin. This chapter reviews recent advances in mitophagy, other possible pathways, and the direction of future research suggested by hypotheses derived from studies of *Drosophila* and cultured *Drosophila* cells.

DROSOPHILA MODELS FOR PARKINSON'S DISEASE

Greene *et al.* (2003) identified a *Parkin* orthologous gene in *Drosophila* and demonstrated that Parkin-deficient flies show severe mitochondrial pathologies of the spermatids and the indirect flight muscles. Massive cell death and the degeneration of mitochondria in the indirect flight muscles and lower electron density in the mitochondrial matrices of both the spermatids and the indirect flight muscles have been observed in *Parkin*-mutant flies. Behavioral studies have shown that decreases in climbing and locomotion abilities are caused by the loss of Parkin function and that these phenotypes are exacerbated by aging. These results appear to be relevant to PD phenotypes. Another study of *Parkin*-null mutant flies showed a reduction in longevity and an increase in sensitivity to oxidative and cold stresses in addition to the degeneration of mitochondria in the muscles. However, the degeneration of dopaminergic neurons in the central nervous system, which is a marker of PD, was not observed, at least in middle-aged adult flies (Pesah *et al.*, 2004).

Clark *et al.* (2006) observed abnormal cell death, degeneration, and fragmentation of mitochondria in the indirect flight muscles and the vacuolation of mitochondria in spermatids in PINK1-deficient flies. These authors found that the loss of PINK1 activity increased the sensitivity to multiple stresses, such as oxidative stress, endoplasmic reticulum stress, and osmotic stress, and reduced the lifespan and the level of ATP. These results indicate that PINK1 and Parkin are essential for the maintenance of the mitochondria, and mitochondrial dysfunction leads to biological vulnerability in *Drosophila*. Mitochondrial pathologies in *PINK1* mutant flies are rescued by the expression of not only *Drosophila PINK1* but also human *PINK1*. Park *et al.* (2006) and Yang *et al.* (2006) reported similar results and observed a reduction in the number of central dopaminergic neurons caused by mitochondrial degeneration.

MITOPHAGY AND MITOCHONDRIAL DYNAMICS

In cultured yeast and mammalian cells, the segregation of abnormal mitochondria from the healthy mitochondrial network through fission processes precedes mitophagy. Twig *et al.* (2008) reported that the depolarization of mitochondria stimulates fission events in cultured cells, which results in the elimination of mitochondria by mitophagy. Studies using cultured *Drosophila* cells and fly models revealed that PINK1 and Parkin regulate mitochondrial fission/fusion events. Drp1 is involved in the fission process, whereas Opa1 and Mitofusin (Mfn) regulate the fusion process. The *Parkin* and *PINK1* phenotypes are suppressed by increased Drp1 activity and are exacerbated by Opa1 or Mfn activities in *Drosophila* (Deng *et al.*, 2008; Poole *et al.*, 2008; Yang *et al.*, 2008). The overexpression of PINK1 causes the degeneration of the compound eyes, and this degeneration is suppressed by the overexpression of Drp1 and enhanced by the overexpression of Opa1 or Mfn (Poole *et al.*, 2008). The morphological phenotypes of mitochondria in *PINK1*- and *Parkin*-mutant flies are also modified by the activity of Drp1, Opa1, or Mfn in the testis, the indirect flight muscles and the dopaminergic neurons of the central nervous system (Deng *et al.*, 2008; Yang *et al.*, 2008). *Drosophila* and cultured *Drosophila* cell studies by Ziviani *et al.* (2010) and

Poole *et al*. (2008) demonstrated that Mfn is ubiquitinated and degraded by the PINK1-Parkin pathway. This process is thought to be one of the mechanisms of the regulation of mitochondrial dynamics by PINK1 and Parkin and appears to be conserved in mammals. Mfn ubiquitination and degradation may prevent the refusion of damaged mitochondria with the healthy mitochondrial population and aid in the isolation and elimination of damaged mitochondria by the autophagy machinery.

MUSCLE DEGENERATION CAUSED BY MITOCHONDRIAL DEFECTS

Animal studies have revealed a physiological function of the PINK1-Parkin pathway in mitochondrial maintenance. Chen and Dorn II (2013) reported that a loss of Mfn2 activity causes cardiomyopathy in mouse and that a loss of Parkin induces cardiomyopathy of the heart tubes in *Drosophila*. PINK1-deficient mice show age-dependent cardiac hypertrophy and increased oxidative stress that is mediated by mitochondrial dysfunction (Billia *et al*., 2011). These studies of *PINK1* and *Parkin* using mice and *Drosophila* (Clark *et al*., 2006; Greene *et al*., 2003) strongly suggest that muscles with higher energy requirements, such as the cardiac muscles and the indirect flight muscles, require the mitochondrial maintenance mediated by the PINK1-Parkin pathway. Although it is believed that cardiomyopathy in PD cases is caused by dysfunctions of the autonomic nervous system, further investigations of mitochondrial dysfunction of the cardiac muscles may provide new insights regarding the relationship between cardiomyopathy and PD phenotypes.

MITOPHAGY AND PROTEIN TRANSLATION SIGNALING

The mitochondrial quality control mechanisms of the PINK1-Parkin pathway may also be affected by energy metabolism and the protein synthesis pathways. In a genetic screening study of *PINK1* mutant flies to identify new components of the PINK1-Parkin pathway, Liu and Lu (2010) determined that S6 kinase (S6K) and eukaryotic translation initiation factor 4E-binding protein (4E-BP), both of which are effectors of Target of rapamycin (TOR), modulate the *PINK1* mutant phenotypes. The TOR pathway regulates protein translation, mRNA transcription, and autophagy (Wullschleger *et al*., 2006), and general protein translation is promoted by S6K and inhibited by 4E-BP. The overexpression of 4E-BP suppresses the mitochondrial dysfunction caused by the loss of PINK1 (Liu and Lu, 2010; Tain *et al*., 2009), whereas the overexpression of S6K exacerbates the *PINK1* phenotype in muscles and dopaminergic neurons (Liu and Lu, 2010). Thus, increased general protein translation aggravates the *PINK1* and *Parkin* phenotypes, likely because protein translation increases cellular energy consumption and leads to increased oxidative stress. Interestingly, the overexpression of an autophagy-related kinase, Atg1, which is negatively regulated by TOR, rescues the *PINK1* mutant phenotype (Liu and Lu, 2010). The rescuing effect of Atg1 on *PINK1* phenotypes is blocked by RNAi against Atg18, which is an additional factor essential for

autophagy, whereas the rescuing effects of the introduction of Parkin are not affected by Atg18 disruption. These results suggest the possibility that two independent mechanisms of mitochondrial quality control are mediated by PINK1, namely, a mitophagy-dependent mechanism and a Parkin-dependent mitophagy-independent mechanism.

CROSSTALK BETWEEN MITOPHAGY SIGNALS AND THE UBIQUITIN-PROTEASOME PATHWAY

Mitophagy is thought to be one of the mechanisms of mitochondrial quality control that is mediated by the PINK1-Parkin pathway. However, recent studies have also suggested the existence of another quality control mechanism that is independent of autophagy. A proteomic and cell biology study revealed that the degradation of mitochondrial outer membrane proteins is regulated by Parkin and the ubiquitin-proteasome pathway (Chan *et al.*, 2011; Sarraf *et al.*, 2013). Vincow *et al.* (2013) measured the turnover of mitochondrial proteins through the metabolic labeling of proteins in *Parkin*- and *Atg7*-mutant flies and provided evidence that the mitophagy-independent degeneration of respiratory chain (RC) proteins is also mediated by the PINK1-parkin pathway. Atg7, which mediates nonselective autophagy, has been reported to act downstream of Parkin in mitophagy in cultured cells. Mutations in *Atg7* and *Parkin* extend the half-life of general mitochondrial proteins in *Drosophila*. The study of Vincow *et al.* (2013) suggests that the turnover of RC proteins depends more strongly on PINK1 and Parkin than on Atg7, and thus RC proteins may be selectively degraded by the PINK1-Parkin pathway, most likely in cooperation with the ubiquitin-proteasome pathway.

Although the GTPases for mitochondrial fusion, Mfn1 and Mfn2, are ubiquitinated and degraded by Parkin and the proteasome, prior degradation of Mfns is not necessary for mitophagy in mammalian cultured cells (Tanaka *et al.*, 2010). Mutations of the VCP/p97 AAA-ATPase cause degeneration of the nervous system, muscles, and bone that lead to the development of amyotrophic lateral sclerosis, parkinsonism, frontotemporal dementia, myopathy, and Paget's disease of bone. Although the pathogenic mechanisms by which VCP mutation leads to these diseases are largely unknown, Kim *et al.* (2013) observed a genetic interaction between VCP and the PINK1-Parkin pathway and revealed that the loss of VCP activity suppresses the Mfn degradation mediated by Parkin and leads to mitochondrial degradation.

A large-scale proteomic study to find candidate targets of Parkin-dependent ubiquitination among mitochondrial and cytoplasmic proteins was recently performed by Sarraf *et al.* (2013). Quantitative diGly capture proteomics were employed in an attempt to reveal proteins whose ubiquitination is Parkin-dependent at endogenous Parkin levels. The Parkin substrates identified by these authors included mitochondrial and cytoplasmic proteins as well as other compartment proteins whose functions include mitochondrial fission/fusion, molecular transport, apoptosis, iron-sulfur shuttling, protein translation and proteasome assembly or activity. These results, together with those from *Drosophila* genetics and cell biology studies, also suggest the existence of a mitochondrial quality control mechanism that is independent of mitophagy (Figure 9.1).

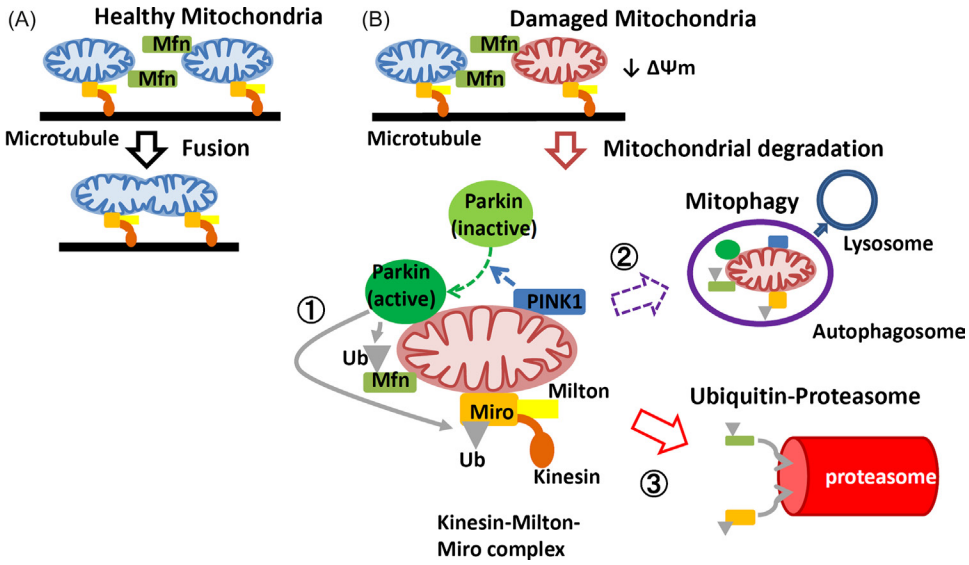

FIGURE 9.1 Model of the mitochondrial quality control mechanism. (A) Kinesin-Milton-Miro complexes transport healthy mitochondria along the microtubule, and mitofusin (Mfn) is involved in the fusion process that is important for the maintenance of a healthy mitochondrial network. (B) When the mitochondrial membrane potential (Δψm) decreases, PINK1 is stabilized and activated on the mitochondrial outer membrane and recruits Parkin from the cytosol to the mitochondria. Mitochondrial Parkin is activated and ubiquitinates mitochondrial proteins such as Mfn and Miro to block the mitochondrial fusion event and mitochondrial transport (1). Ubiquitinated mitochondria are catabolized by the mitophagy machinery (2). Recent *Drosophila* studies suggest a revised model of mitochondrial quality control in which unfolded or damaged mitochondrial proteins are constitutively degenerated by the ubiquitin-proteasome machinery together with the PINK1-Parkin pathway (3).

POSSIBLE ROLES OF MITOPHAGY IN NEURONS

Whether PINK1 and Parkin regulate mitophagy under physiological conditions *in vivo*, especially during neuronal activities that are associated with PD, is largely unknown. Mitochondria in neurons are shuttled between the cell body and the axon terminals via microtubule-dependent transport involving motor-adaptor complexes of kinesin/dynein motors and Miro/Milton. Wang *et al.* (2011) reported that Parkin, in cooperation with PINK1, breaks down the Miro component of the mitochondrial motor-adaptor complex via ubiquitination in mammalian cultured hippocampal neurons and *Drosophila* larvae motor neurons, and this process results in the suppression of mitochondrial motility. Liu *et al.* (2012) also observed similar results in *Drosophila* larvae motor neurons, and these authors found that the overexpression of Miro results in mitochondrial aggregation and cell death in the dopaminergic neurons of the brain. Biochemical and cell culture studies have suggested that the degradation of Miro by Parkin facilitates Parkin-mediated mitophagy upon depolarization of the mitochondrial membrane potential. These studies propose the concept that the degradation of Miro by PINK1 and Parkin ensures the transport of healthy mitochondria to the synaptic terminals and arrests the movement of the damaged mitochondria on which PINK1 should be activated.

REGULATORS OF MITOCHONDRIAL MAINTENANCE IDENTIFIED IN *DROSOPHILA* STUDIES

Drosophila genetic studies using *PINK1*- and *Parkin*-mutant flies have successfully identified several new candidate regulators and signaling pathways. Phosphoglycerate mutase 5 (PGAM5), which is localized at the outer mitochondrial membrane, was identified as a PINK1-binding protein by Imai *et al*. (2010). A loss of PGAM5 improves the mitochondrial phenotypes and extends the shortened lifespan caused by the loss of PINK1 activity in *Drosophila*, whereas the overexpression of PGAM5 induces fragmentation and degeneration of mitochondria in the indirect flight muscles. However, PGAM5 inhibition does not affect the shortened lifespan or the abnormal phenotypes of mitochondria in *Parkin*-mutant flies. These results led to the proposal of two hypotheses regarding the relationship between PGAM5 and the PINK1-Parkin pathway: PGAM5 is downstream of PINK1 and negatively regulates Parkin, or PGAM5 plays a role in mitochondrial maintenance as an effector of PINK1 that is independent of Parkin.

Mutations of the *Rad6a/Ube2a* gene cause X-linked intellectual disability in humans, and *Rad6a*-deficient *Drosophila* show mitochondrial failure and synaptic dysfunction (Haddad *et al*., 2013). Rad6a acts as an E2 ubiquitin-conjugating enzyme for Parkin upon the mitophagy of damaged mitochondria by forming Lys48- and Lys63-linked poly-ubiquitination chains of mitochondrial proteins.

Esposito *et al*. (2013) screened for modifiers of *PINK1*-mutant flies in a mutant fly collection with mitochondrial phenotypes and identified that loss-of-function mutations of the *Aconitase* gene suppress *PINK1* mutant phenotypes. Aconitase is an iron-sulfur protein that is involved in the first step of the tricarboxylic acid (TCA) cycle, and oxidative inactivation of its iron-sulfur cluster by superoxide leads to iron toxicity and peroxide production. The loss of *PINK1* activity results in mitochondrial failure including complex I dysfunction, which may generate toxic superoxides, leading to *Aconitase* inactivation. This mechanism is thought to be independent of Parkin-mediated mitophagy because *Aconitase* inactivation fails to rescue *Parkin* mutant phenotypes in *Drosophila*.

Wu *et al*. (2013) screened another modifier of *PINK1* mutant phenotypes in *Drosophila* and identified the TORC2 pathway. In this study, epistasis analyses suggested that the TORC2 and Tricornered (Nuclear dbf2-related kinase in mammals) kinases act downstream of PINK1 and upstream of Parkin to regulate mitochondrial integrity in *Drosophila*, and this mechanism appears to be conserved in mammals mostly.

TOOLS FOR THE DETECTION OF MITOPHAGY IN *DROSOPHILA*

Although Parkin-mediated mitophagy has been extensively characterized in cultured cells, the extent to which mitophagy contributes to physiological events *in vivo* continues to be debated. Because *in vivo* detection techniques for autophagy via imaging have been well developed, these techniques could be applied to monitor mitophagy *in vivo*. The fusion protein of the microtubule-associated protein light chain 3 (LC3) and EGFP (LC3-EGFP) has been shown to be a highly specific probe for the formation of the isolation membrane during the

TABLE 9.1 Summary of *Drosophila* Model Studies on the Mechanisms of Mitochondrial Quality Control

Gene	Material/Tissue	Findings	References
Parkin	Indirect flight muscle Testis DA neuron Heart tube	Mutants induce: massive cell death mitochondrial degeneration decrease in the climbing and locomotion abilities reduced lifespan sensitivity to oxidative and cold stresses cardiomyopathy of heart tube	Greene *et al.*, 2003 Pesah *et al.*, 2004 Chen and Dorn II, 2013
PINK1	Indirect flight muscle Testis DA neuron	Mutants induce: massive cell death mitochondrial degeneration and fragmentation decrease in the climbing and flight abilities decrease ATP levels reduced lifespan sensitivity to multiple stresses	Clark *et al.*, 2006 Park *et al.*, 2006 Yang *et al.*, 2006
	Indirect flight muscle		Poole *et al.*, 2008
Mfn	Eye	Mitochondrial fission (Drp1) and fusion (Mfn, Opa1) machinery modulates PINK1-Parkin pathway	Yang *et al.*, 2008
Opa1	DA neuron	Mfn is ubiquitinated and degenerated by PINK1-Parkin pathway	Deng *et al.*, 2008
Drp1	Cultured cell		Ziviani *et al.*, 2010 Poole *et al.*, 2008
4E-BP	Indirect flight muscle	4E-BP suppresses the PINK1 phenotypes	Tain *et al.*, 2009
S6K	DA neuron	S6K exacerbates the PINK1 phenotypes	Liu and Lu, 2010
Miro	Larval motor neuron	Kinesin-Milton-Miro complex transfers mitochondria from cytosol to synapse	Wang *et al.*, 2011
	DA neuron	Miro is ubiquitinated and degenerated by the PINK1-Parkin pathway	Liu *et al.*, 2012
PGAM5	Indirect flight muscle DA neuron	Loss of PGAM5 alleviates the PINK1 phenotypes	Imai *et al.*, 2010
Rad6a/Ube2a	Larval neuromuscular junction	E2 ubiquitin-conjugating enzyme, Rad6a-deficient fly shows mitochondrial failure and synaptic dysfunction	Haddad *et al.*, 2013
Aconitase	Indirect flight muscle Da neuron	Aconitase mutants suppress *PINK1* mutant phenotype and this mechanism is independent of Parkin	Esposito *et al.*, 2013
TORC2 *Tricornered*	Indirect flight muscle DA neuron	TORC2 and Tricornered kinases act downstream from PINK1 but upstream of Parkin	Wu *et al.*, 2013

DA neuron: Dopaminergic neuron.

activation of the autophagy process; this probe can also be used as a marker of mitophagy (Kabeya *et al*., 2000; Mizushima *et al*., 2001). Katayama *et al*. (2011) developed a new technique for the detection of mitophagy in cultured cells that employs the unique red fluorescent protein Keima; the emission profile of Keima includes a pH-dependent wavelength shift. When mitochondria labeled with mitochondria-targeting signal-conjugated Keima are delivered to the lysosomes via Parkin-mediated mitophagy, the acidic pH of the lysosomes alters the fluorescence emission ratio induced by two different excitation wavelengths. This technique could be applied to the observation of mitophagy in living *Drosophila* using live imaging.

Pimenta de Castro *et al*. (2012) generated a *Drosophila* model to understand protein conformational stress in mitochondria. Ubiquitous expression of a mutant form of the mitochondrial matrix protein ornithine transcarbamylase was achieved using a tissue-specific GAL4-UAS expression system in *Drosophila* phenocopies of *PINK1*- and *Parkin*-mutant flies. Abnormal mitochondrial morphologies in the indirect flight muscles, reductions in climbing ability, and decreased life span are observed in these flies, and these phenotypes are alleviated by the enhancement of PINK1 and Parkin signaling. This model could genetically induce mitophagy in flies without the use of mitochondria-damaging reagents such as cyanide m-chlorophenylhydrazone (CCCP).

CONCLUDING REMARKS

Although oxidative stress due to mitochondrial dysfunction is generally believed to contribute to the etiology of PD, conclusive evidence has now emerged from a series of *Drosophila* genetic studies (Table 9.1) that mitochondrial dysfunction is one of the major causes of young-onset familial PD linked to *PINK1* and *Parkin*. Elegant cell biology studies have demonstrated that the PINK1-Parkin pathway regulates the selective autophagy of mitochondria (i.e., mitophagy) via the use of mitochondrial uncouplers such as CCCP. However, strong evidence that mitophagy occurs in neurons associated with PD in physiological conditions remains lacking. On the other hand, recent *Drosophila* studies have suggested that the PINK1-Parkin pathway is implicated in the selective degradation of RC proteins and the unfolding of mitochondrial proteins. However, there is no direct, molecular-level evidence from cell culture studies. Further insights from both cell biology and animal model studies, including powerful *Drosophila* model studies, will elucidate the mitochondrial quality control mediated by the PINK1-Parkin pathway (Figure 9.1).

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CHAPTER

10

Atg32 Confers Selective Mitochondrial Sequestration as a Cargo for Autophagy

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OUTLINE

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Abstract

Mitochondria are organelles that supply a large amount of energy required for cellular activities. Accumulation of dysfunctional mitochondria within cells inhibits cellular functions and causes several diseases. Thus, the cell has devised specific mechanisms to ensure proper quality and quantity control of this organelle. Mitochondrial autophagy (mitophagy) is thought to be one of the primary mechanisms for mitochondrial quality control that selectively eliminate dysfunctional or excess mitochondria via an autophagic process. *ATG32* is a mitophagy-specific gene identified by a yeast genome-wide screen for mitophagy. Atg32, a protein encoded by *ATG32*, is a transmembrane protein localized in the mitochondrial outer membrane. During mitophagy induction, Atg32 functions as a mitochondrial receptor protein that interacts with cytosolic adaptor protein Atg11, which recruits mitochondria to the autophagic machinery for degradation. In this chapter, we describe the molecular mechanism and physiology of mitophagy in yeast, with an emphasis on the role of Atg32.

INTRODUCTION

Autophagy is a conserved degradative pathway that delivers cytoplasmic components to lysosomes in mammalian cells or vacuoles in yeast, for degradation. Typically, autophagy is induced in response to various types of cellular stress to degrade cytoplasmic proteins and organelles nonselectively. Autophagy also plays diverse roles in cellular development, tumor suppression, immune responses, and aging, and in the prevention of many diseases such as infection, neurodegenerative disease, cardiomyopathy, and diabetes in mammalian cells. Although it was initially thought that autophagy was a nonselective degradative pathway, recent studies revealed that a selective type of autophagy targets specific cellular components for degradation. It is now known that mitochondria, peroxisomes, ribosomes, endoplasmic reticulum, lipid droplets, protein aggregates, and invasive microbes are recognized as selective autophagic cargo and these selective autophagic processes are termed mitophagy (Lemasters, 2005), pexophagy (Scott and Klionsky, 1998), ribophagy (Kraft *et al*., 2008), ERphagy (Bernales *et al*., 2007), lipophagy (Singh *et al*., 2009), aggrephagy (Ding *et al*., 2007; Komatsu *et al*., 2007), and xenophagy (Levine, 2005), respectively.

The mitochondrion is an organelle that supplies a large amount of energy using an electron transport chain and oxidative phosphorylation, and contributes to a range of cellular activities. However, this process also generates reactive oxygen species (ROS) as a natural byproduct. ROS causes oxidative damage to mitochondrial lipids, DNA, and proteins. Damaged mitochondria generate increased levels of ROS, which in turn damages mitochondria more severely. To prevent this vicious cycle, appropriate quality and quantity control of mitochondria is important. Accordingly, the cell has evolved specific mechanisms to ensure proper quality and quantity control of mitochondria. Mitophagy is thought to be one of the primary mechanisms for mitochondrial quality control that selectively eliminates dysfunctional or excess numbers of mitochondria. There has been significant progress in the study of mitophagy in recent years and our understanding of the molecular mechanisms and physiological roles of mitophagy has greatly increased. During erythroid cell maturation of mammalian cells, the mitochondrial outer-membrane protein Nix mediates selective mitochondrial elimination (Sandoval *et al*., 2008; Schweers *et al*., 2007). Loss-of-function mutations of the *PARK2* and *PARK6* genes, which encode Parkin and PINK1, respectively,

cause Parkinson disease. Parkin and PINK1 can stably localize to impaired mitochondria and promote autophagic degradation of the impaired mitochondria (Narendra *et al.*, 2008, 2010). These findings suggest that mitophagy is involved in cellular physiology and disease. Despite the importance of mitophagy, the molecular mechanism and regulation of mitophagy in mammalian cells are still not fully elucidated. In contrast, they have been relatively well documented in yeast (Ding and Yin, 2012). In this chapter, we introduce the molecular mechanism and physiology of mitophagy in yeast, with an emphasis on a protein indispensable for mitophagy, Atg32.

Atg32, A MITOPHAGY-SPECIFIC PROTEIN

Genome-wide Screen for Mitophagy-Deficient Mutants in Yeast

To date, numerous autophagy studies have identified 36 AuTophaGy-related (*ATG*) genes in yeast. At least 15 of these genes are required for both nonselective and selective autophagy. Thus, these genes are thought to encode core factors for autophagy. Because mitophagy is a type of autophagy, most core factors of autophagy are required for mitophagy. To identify factors specifically required for mitophagy, two groups used nonessential gene deletion yeast strains and performed a genome-wide screen for mutants that were defective in mitophagy (Kanki *et al.*, 2009a; Okamoto *et al.*, 2009). Okamoto *et al.* (2009) examined 5150 knockout strains and found 36 mutants that impaired mitophagy, excluding existing *ATG* gene null strains. Similarly, Kanki *et al.* (2009a) observed 4667 knockout strains and found 32 mutants defective in mitophagy, again excluding existing *ATG* gene null mutants. Although both groups used similar methods to observe mitophagy, only 35% of identified genes were common between the two screens, presumably because of the different mitophagy-inducing conditions used. Both groups focused on *ATG32*, which is now known to encode an indispensable mitophagy-related protein Atg32, and analyzed Atg32 in detail (Kanki *et al.*, 2009b; Okamoto *et al.*, 2009). The deletion of *ATG32* does not affect nonselective autophagy and pexophagy, but completely inhibits mitophagy, suggesting *ATG32* is a mitophagy specific gene. *ATG32* does not have obvious homologues in higher eukaryotes, but does have putative homologues in other fungi including *Candida glabrata*, *Kluyveromyces lactis*, and *Eremothecium gossypii* (Kanki *et al.*, 2009b; Okamoto *et al.*, 2009).

Localization and Topology of Atg32

Atg32 is composed of 529 amino acids (~59-kDa) and is predicted to have a single transmembrane domain. Green fluorescent protein-tagged Atg32 colocalizes with mitochondrial markers. A proteinase sensitivity assay conducted on crude mitochondrial fractions suggested that Atg32 is located in the mitochondrial outer membrane with its N- and C-terminal domains oriented towards the cytosol and the mitochondrial intermembrane space, respectively (Kanki *et al.*, 2009b; Okamoto *et al.*, 2009). The N-terminal cytosolic region of Atg32 contains Atg8- and Atg11-interacting regions and is indispensable for mitophagy, while the C-terminal mitochondrial intermembrane space region is dispensable

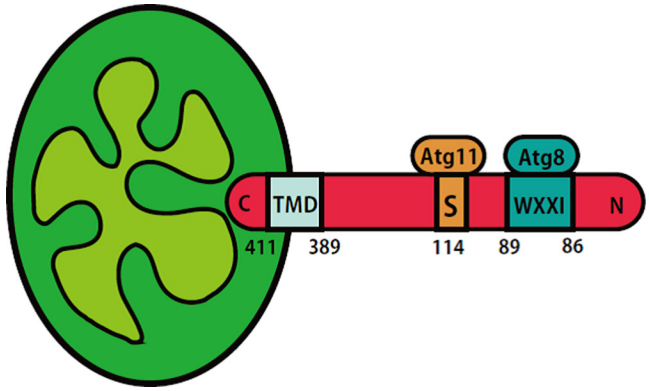

FIGURE 10.1 The primary structure of Atg32. Atg32 consists of 529 amino acids with a single transmembrane domain (TMD). Atg32 is located in the mitochondrial outer membrane with the N- and C-terminal domains oriented towards the cytosol and the mitochondrial intermembrane space, respectively. The N-terminal cytosolic region of Atg32 contains Atg8 and Atg11 interacting regions, containing WxxI sequence and serine 114, respectively.

for mitophagy (Aoki *et al.*, 2011; Kondo-Okamoto *et al.*, 2012). The transmembrane domain of Atg32 is essential for its localization and integration into the mitochondrial outer membrane (Aoki *et al.*, 2011; Kondo-Okamoto *et al.*, 2012). Figure 10.1 summarizes the primary structure of Atg32.

Atg32 INTERACTS WITH ATG11 FOR SELECTIVE MITOCHONDRIAL DEGRADATION

Atg11, an Adaptor Protein for Selective Autophagy

Atg11 is a cytosolic adaptor protein for selective autophagy. Initially, Atg11 was identified as a component specifically required for the cytoplasm-to-vacuole targeting (Cvt) pathway (Kim *et al.*, 2001). The Cvt pathway is observed only in yeast and is a selective autophagy-like process that delivers Cvt complex (aminopeptidase I and alpha-mannosidase complex) and Ape4 from the cytoplasm to vacuoles without delivering any additional known cargo. It has now been shown that Atg11 is required for selective autophagy including the Cvt pathway, pexophagy, and mitophagy, but not nonselective autophagy (Kanki and Klionsky, 2008; Kim *et al.*, 2001; Mukaiyama *et al.*, 2002). Atg11 is composed of 1178 amino acids (~135-kDa) and has four coiled-coil domains. Atg11 interacts with cargo-specific receptor proteins and selectively recruits cargo to the pre-autophagosomal structure/phagophore assembly site (PAS), where sequestering cytosolic vesicles are generated. For example, during the Cvt pathway, Atg11 interacts with Atg19, a specific receptor protein of the Cvt complex, and recruits the Cvt complex to the PAS (Shintani *et al.*, 2002). Similarly, during pexophagy in *Pichia pastoris*, PpAtg30, a receptor protein, localizes to peroxisomes, where it subsequently binds PpAtg11, allowing recruitment of peroxisomes to the PAS (Farre *et al.*, 2008).

Atg32 is a Receptor Protein for Mitophagy that Interacts with Atg11

Because Atg32 is a mitophagy-specific protein and localizes in the mitochondrial outer membrane, Atg32 was speculated to be a receptor protein for mitophagy. To examine this possibility, the two groups who had identified Atg32 performed yeast two-hybrid and immunoprecipitation experiments and found that Atg32 could interact with Atg11. Notably, Atg32–Atg11 interactions dramatically increased under mitophagy inducible conditions (Kanki *et al.*, 2009b; Okamoto *et al.*, 2009). These findings suggest that, similarly with the Cvt pathway and pexophagy, the cytosolic adaptor protein Atg11 binds to the mitochondrial receptor protein Atg32, and then Atg11 recruits mitochondria to the PAS for autophagic degradation. We further studied the Atg11–Atg32 interaction in detail. Yeast two-hybrid and immunoprecipitation experiments revealed that the C-terminus region of Atg11, which included the fourth coiled-coil domain, interacted with the N-terminus region of Atg32 (residues 100–120) (Aoki *et al.*, 2011).

Phosphorylation of Atg32 Mediates Atg32–Atg11 Interactions and Mitophagy

We observed the molecular weight of Atg32 increased when mitophagy was induced, and analyzed the mechanism of molecular weight shift of Atg32. We found that Atg32 was phosphorylated when mitophagy was induced and that the phosphorylation site included serines 114 and 119 on Atg32 (Aoki *et al.*, 2011). Because serines 114 and 119 on Atg32 are within the Atg11 interacting residues, we speculated that phosphorylation of serine 114 and/or 119 is important for Atg32–Atg11 interactions. As we expected, a serine 114 to alanine mutant of Atg32 did not interact with Atg11, and as a result could not induce mitophagy, while a serine 119 to alanine mutant of Atg32 marginally affected mitophagy (Aoki *et al.*, 2011). From these findings, we concluded that when mitophagy is induced, serines 114 and 119 on Atg32 are phosphorylated, and that phosphorylation of Atg32, especially on Serine 114, mediates the Atg32–Atg11 interaction and mitophagy (Figure 10.2).

Atg32 Interacts with Atg8

Atg8 is a ubiquitin-like protein conjugated to the lipid phosphatidylethanolamine (PE), and localizes to the autophagic sequestering double-membrane (called phagophore or isolation membrane), and is involved in phagophore expansion. Atg8 and its mammalian homologue LC3 interact with a WxxL-like sequence that is typically observed in autophagy receptor proteins, such as Atg19 in yeast and p62, NBR1, and Nix in mammalian cells. Atg32 also has a similar WxxI (WQAI) sequence in residues 86 to 89. It was also demonstrated that Atg8 interacts with Atg32 through the WxxI sequence using a yeast two-hybrid assay, immunoprecipitation assays, and crystal structure analysis (Kondo-Okamoto *et al.*, 2012; Okamoto *et al.*, 2009). Mutation of the WxxI sequence in Atg32 severely affected Atg32–Atg8 interactions, but did not affect Atg32–Atg11 interactions (Okamoto *et al.*, 2009). Thus, the Atg32–Atg8 interaction is not essential but is important for mitophagy efficiency (Kondo-Okamoto *et al.*, 2012).

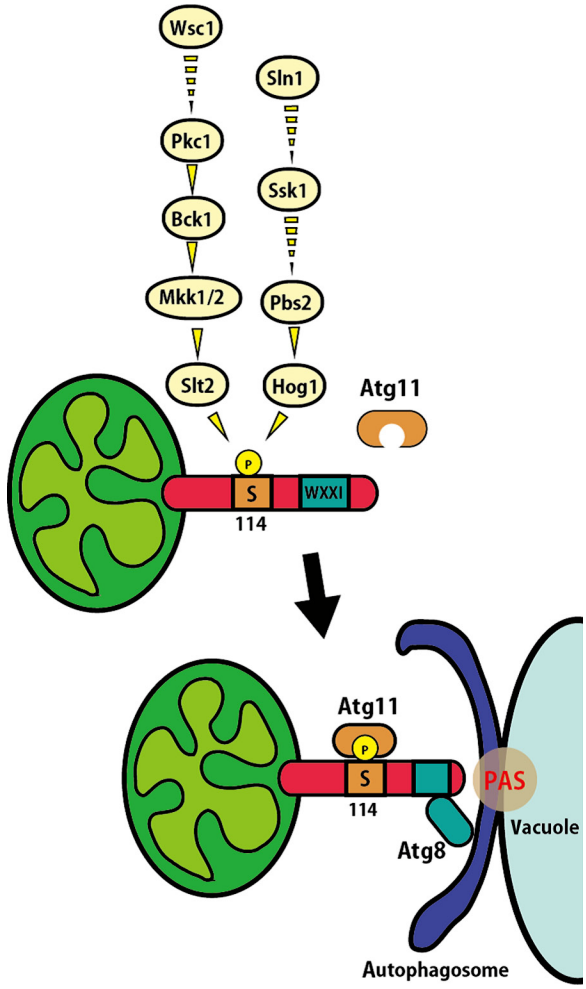

FIGURE 10.2 Schematic model of mitophagy in yeast. Environmental factors stimulate cell surface stress sensors, Wsc1 and Sln1, which subsequently activate Slt2 and Hog1 signaling pathways. Eventually, serines 114 and 119 on Atg32 are phosphorylated and phosphorylation of Atg32, particularly at serine 114, mediates the Atg11–Atg32 interactions. Atg11 recruits mitochondria to the phagophore assembly site (PAS) where the autophagosome is generated to enclose the mitochondria, which is then delivered to the vacuole and degraded.

INDUCTION AND REGULATION OF MITOPHAGY

Mitophagy Induction in Yeast

Mitophagy is rarely induced in yeast *Saccharomyces cerevisiae* under the common culture conditions used by most laboratories (or even if induced, it cannot be detected). At present, we know very few conditions that can induce mitophagy efficiently in wild-type yeast cells. Mitophagy is efficiently induced by nitrogen starvation after preculturing yeast in a

nonfermentable medium that facilitates the proliferation of mitochondria (e.g., where lactate or glycerol is the sole carbon source). In addition, mitophagy is efficiently induced during the stationary phase when yeast cells are cultured in a nonfermentable medium (Kanki and Klionsky, 2008; Kissova *et al*., 2004; Tal *et al*., 2007). Although macroautophagy is also activated under these mitophagy-inducing conditions, mitochondria are specifically selected and degraded by mitophagy via Atg11–Atg32 interactions.

In mammalian cells, there is accumulating evidence that dysfunctional (or depolarized) mitochondria are selectively eliminated by mitophagy (Narendra *et al*., 2008; Twig *et al*., 2008). However, there is limited evidence that damaged mitochondria are eliminated by mitophagy in yeast. For example, interference with F_0F_1-ATPase biogenesis in a temperature-sensitive *fmc1* deletion mutant, or osmotic swelling of mitochondria caused by depletion of the mitochondrial K^+/H^+ exchanger Mdm38 induces mitophagy (Nowikovsky *et al*., 2007; Priault *et al*., 2005). On the other hand, mitochondrial depolarization caused by an uncoupler such as carbonyl cyanide m-chlorophenylhydrazone (CCCP) does not induce mitophagy in yeast (Kanki *et al*., 2009b; Kissova *et al*., 2004).

Signaling Pathways that Regulate Mitophagy

Two mitogen-activated protein kinases (MAPKs), Hog1 and Slt2, are related to mitophagy (Aoki *et al*., 2011; Mao *et al*., 2011). Hog1 is a kinase integral to the osmoregulatory signal transduction cascade (HOG signaling pathway). Mao *et al*. (2011) analyzed upstream molecules of the HOG signaling pathway to determine if it was related to mitophagy. They found that Sln1, a sensor in the plasma membrane, Ssk1, a cytoplasmic response regulator downstream of Sln1, and Pbs2, a MAPK kinase just upstream of Hog1, were related to mitophagy (Mao *et al*., 2011). Slt2 is a kinase involved in the cell-wall-integrity pathway. They analyzed upstream molecules of the Slt2 pathway and found that Wsc1, a cell surface stress sensor, protein kinase C (Pkc1), MAPK kinase kinase (Bck1), and MAPK kinase (Mkk1/Mkk2) were related to mitophagy. The downstream factors in both pathways have not been identified. As mentioned above, phosphorylation of Atg32 is the initial step of mitophagy. Thus, Hog1 and/or Slt2 may phosphorylate Atg32 and regulate mitophagy. Indeed, *hog1*-deleted yeast strongly inhibits Atg32 phosphorylation under mitophagy inducing conditions. However, by *in vitro* experiment, the direct phosphorylation of Atg32 by Hog1 could not be observed (Aoki *et al*., 2011). This finding suggests that although the HOG signaling pathway has an important role in mitophagy by regulating Atg32 phosphorylation, there may be unidentified kinase(s) that directly phosphorylate Atg32 downstream of Hog1. Mao *et al*. (2011) speculated that Slt2 signaling was activated in the earlier stages of mitophagy in comparison to Hog1 signaling. However, it was not determined whether Slt2 could directly phosphorylate Atg32. Figure 10.2 summaries the current molecular process of mitophagy in yeast.

OTHER FACTORS RELATED TO MITOPHAGY IN YEAST

In addition to Atg32, other proteins such as Uth1, Aup1, Atg33, and Whi2 have been reported to be required for mitophagy. Uth1, a mitochondrial outer membrane-localizing SUN family protein, was identified as a mitophagy-related protein in yeast (Kissova *et al*., 2004).

uth1-deleted yeast strains show partial inhibition of mitophagy; these strains are blocked at an early stage of mitophagy (Kissova *et al.*, 2007). However, how Uth1 relates to mitophagy has not been clarified.

Aup1 was identified by a screen for protein phosphatase homologues that interact with the serine/threonine kinase Atg1 required for autophagy. Aup1 was suggested to be required for efficient mitophagy to survive prolonged stationary phase culture in a medium containing a nonfermentable carbon source (Tal *et al.*, 2007). In addition, it was shown that deletion of *RTG3*, a transcription factor that mediates the retrograde signaling pathway, causes a defect in stationary phase mitophagy, and that deletion of *AUP1* leads to alterations in the patterns of Rtg3 phosphorylation under these conditions. This implies that the function of Aup1 in mitophagy may be the regulation of Rtg3-dependent transcription (Journo *et al.*, 2009).

Atg33 is a mitophagy-related protein identified by a genetic screen for yeast mutants defective in mitophagy, and is located in the mitochondrial outer membrane (Kanki *et al.*, 2009a). Interestingly, in *atg33*-deleted yeast strains, although starvation-induced mitophagy was partially inhibited, it was severely inhibited when induced during the stationary phase. The function of Atg33 in mitophagy is still unknown. It was suggested that Atg33 might be a factor for the selection or detection of damaged or aged mitochondria when cells have reached the stationary phase (Kanki *et al.*, 2009a).

Whi2 is a stress response protein. Mendl *et al.* (2011) observed that deletion of *WHI2* reduced mitophagy by about 40%, while nonselective autophagy was barely affected. Although how Whi2 relates to the mitophagy has not been clarified, they speculated that Whi2 and the Ras/PKA (protein kinase A) signaling pathway were linked to the regulation of mitophagy.

Cellular oxidative stress is thought to affect mitophagy positively. Deffieu *et al.* (2009) observed that N-acetylcysteine (NAC) and cysteine, which increase cellular levels of reduced glutathione, prevented mitophagy. Okamoto *et al.* (2009) reported that NAC suppressed the expression of Atg32 and as a result, inhibited mitophagy. These findings suggest that Atg32 expression and mitophagy are affected by cellular oxidative conditions. Because mitophagy is thought to eliminate damaged mitochondria preferentially, it is reasonable that cellular oxidative status, caused by ROS generated by damaged mitochondria, may be related to the induction of mitophagy.

PHYSIOLOGICAL ROLE OF MITOPHAGY IN YEAST

It has been suggested that mitophagy eliminates dysfunctional mitochondria, thereby maintaining mitochondrial quality. However, there has been limited evidence to support this idea in yeast. In contrast, a mitophagy-deficient *atg32*-deleted strain did not show any phenotype regarding cell growth, viability, and mitochondrial functions under normal culture conditions (Kanki *et al.*, 2009b).

We recently uncovered part of the physiological role of mitophagy in yeast (Kurihara *et al.*, 2012). When wild-type yeast cells are grown in glucose-rich conditions, cells preferentially ferment glucose to obtain ATP and release ethanol. Under these conditions, limited numbers of mitochondria are present within the cells. When cells absorb glucose,

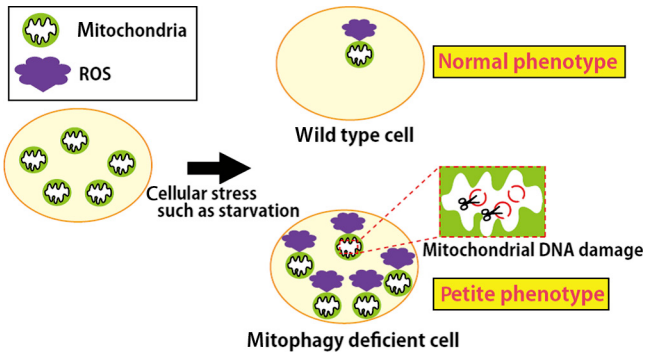

FIGURE 10.3 The importance of mitophagy in yeast. When cells encounter cellular stress such as nitrogen starvation, wild-type cells initiate mitophagy and eliminate excess mitochondria, which produce ROS. In mitophagy-deficient cells, the remaining mitochondria produce excess ROS that in turn can damage mitochondria. Eventually, ROS damaged mitochondrial DNA and cells with damaged mitochondrial DNA are termed the petite phenotype.

they switch metabolism from fermentation to respiration and the aerobic usage of ethanol. Under these conditions, mitochondria proliferate in cells for efficient respiration. In this way, cells switch metabolism between fermentation and respiration (this conversion is called the diauxic shift) and depending on the type of metabolism, the number of cellular mitochondria increases or reduces. Mitophagy-deficient *atg32*-deleted cells could not adequately regulate the number of mitochondria present demonstrated by mitochondrial dysfunction phenotypes. To be more specific, when *atg32*-deleted cells were precultured in nonfermentable medium and were then shifted to nitrogen starvation for long-term culture (~5 days), they generated small colonies when grown on nutrient-rich plates, while wild-type cells did not. Further analysis revealed that, when wild-type cells encounter nitrogen starvation, they induce mitophagy and quickly eliminate mitochondria that have proliferated during respiratory growth. As a result, cellular ROS production, which is mainly caused by mitochondria, is suppressed. However, in *atg32*-deleted cells, undegraded mitochondria produce excess ROS during nitrogen starvation. ROS damages mitochondria, and damaged mitochondria produce further ROS, finally leading to mitochondrial (mt) DNA deletion. Ultimately, cells with mtDNA deletion generate small colonies even in fermentable medium and this phenotype is called "petite" (Kurihara *et al.*, 2012) (Figure 10.3). This suggests that mitophagy is required for regulation of the number of mitochondria to minimize ROS production and, as a result, maintains the quality of mitochondria.

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CHAPTER

11

PARK2 Induces Autophagy Removal of Impaired Mitochondria via Ubiquitination

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OUTLINE

Abstract

Mutations of *PARK2*, a gene encoding the ubiquitin ligase Parkin, are causative of autosomal recessive parkinsonism, a neurodegenerative disorder that is characterized by the relentless loss of dopaminergic neurons in the midbrain. The pivotal role that Parkin plays in maintaining dopaminergic neuronal survival is underscored by our current recognition that its dysfunction not only represents a prevalent cause of familial parkinsonism but also a formal risk factor for the more common, sporadic form of Parkinson's disease (PD). Accordingly, keen research on Parkin over the past decade or so has led to a significant advancement

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in our knowledge regarding its physiological roles and its relevance to PD. In particular, a recent seminal discovery that identified Parkin as a key regulator of mitochondrial quality control has provided an attractive mechanism that potentially underlies mitochondrial dysfunction commonly seen in the PD brain, which is thought to be a pathogenic driver of the disease. Indeed, the finding has led to a flurry of activity to elucidate the precise molecular events underlying Parkin-mediated mitophagy that ensues to this date. However, like all newly proposed models, the current model of Parkin-mediated mitophagy remains imperfect and is continually being debated and updated. In this chapter, we shall discuss the current knowledge and controversies surrounding this exciting topic.

INTRODUCTION

Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder after Alzheimer's disease, with a prevalence of 1–2% in the population above the age of 65. Clinically, the disease is attended by a constellation of motoric deficits including bradykinesia (slowness in movements), postural instability, rigidity, and tremor that progressively worsen with age, eventually leading to near total immobility. Although pathological changes are distributed in the PD brain, the principal neuropathology that underlies the characteristic motor phenotype of PD patients is unequivocally the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) of the midbrain, which normally innervates the striatum (Braak *et al.*, 2003). Accompanying this pathology is the presence of intraneuronal protein inclusions known as Lewy bodies (LBs) in affected regions of the PD brain, which occur in numbers that far exceed their occasional presence in the normal brain. The SNpc neuronal loss results in a severe depletion of striatal dopamine (DA) and thereby an impaired nigrostriatal system that otherwise allows an individual to execute proper, coordinated movements. Accordingly, pharmacological replacement of brain DA via L-DOPA administration represents an effective symptomatic recourse for the patient (especially during the initial stages of the disease) and remains a clinical gold standard treatment for PD. However, neither L-DOPA nor any currently available therapies can slow or stop the insidious degenerative process in the PD brain. Thus, PD remains an incurable disease. According to a recent report, more than 4 million individuals in Europe's five most and the world's ten most populous countries are currently afflicted with PD. In less than 20 years' time (i.e., in 2030), the number of PD sufferers is projected to increase to close to 10 million (Dorsey *et al.*, 2007). This is clearly a worrying trend and one that aptly emphasizes the urgency to develop more effective treatment modalities for the PD patient. Towards this endeavour, a better understanding of the molecular mechanism(s) that underlies the pathogenesis of PD would certainly be helpful, as the illumination of this would allow the identification and therapeutic exploitation of key molecules/events involved in the pathogenic process.

Although a subject of intense research, the etiology of PD unfortunately remains incompletely understood. However, a broad range of studies conducted over the past few decades, including epidemiological, genetic, and postmortem analysis, as well as *in vitro* and *in vivo* modeling, have contributed significantly to our understanding of the pathogenesis of the disease. In particular, the recent identification and functional characterization of several genes, including *PARK2* and a related gene known as *PINK1*, whose mutations are

causative of rare familial forms of PD have provided tremendous insights into the molecular pathways underlying dopaminergic neurodegeneration. Notably, a pathogenic culprit that consistently surfaced from these studies is mitochondrial dysfunction. Given this and our appreciation that mitochondria are dynamic structures that constantly undergo regulated remodeling and turnover (to ensure that they are bioenergetically competent at all times), it is tempting to propose that defective mitochondrial quality control (QC) may underlie dopaminergic neurodegeneration in PD.

MITOCHONDRIAL DYSFUNCTION AND PARKINSON'S DISEASE

The idea that mitochondrial dysfunction could contribute to the development of PD is actually not new. It originally surfaced in the early 1980s after Langston and colleagues noticed that drug abusers exposed to 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP), an inhibitor of mitochondrial complex I function, display motoric features that bear uncanny resemblance to those exhibited by sporadic PD patients (Langston *et al.*, 1983). Thereafter, several groups conducted postmortem analysis of PD brains and recorded a significant reduction in the activity of mitochondrial complex I as well as ubiquinone (co-enzyme Q10) in the SN of diseased samples (Schapira *et al.*, 1989; Shults *et al.*, 1997). Consistent with the proposed role of mitochondrial dysfunction as a key pathogenic driver of PD, mitochondrial poisoning through the administration of toxins such as MPTP and rotenone recapitulates PD-related features in animals (including nonhuman primates) and represents a popular strategy to model the disease (Dauer and Przedborski, 2003). Interestingly, whereas dopaminergic neurodegeneration induced by MPTP can be explained by the fact that its conversion into its toxic principle MPP+ endows it with the selectivity for dopaminergic neurons (by virtue of the exquisite affinity MPP+ has for dopamine transporters), rotenone by comparison is more broadly distributed in the brain following its administration into animals. Despite the more systemic distribution of rotenone in treated animals, its toxicity is mostly confined to dopaminergic neurons, suggesting that dopaminergic neurons are uniquely susceptible to complex I inhibition. More recently, a genetic mouse model of mitochondrial dysfunction known as "MitoPark" was generated via the conditional ablation of TFAM in dopaminergic neurons (Sterky *et al.*, 2011). TFAM is a mitochondrial transcription factor that plays a critical role in maintaining mitochondrial DNA. Similarly to toxin-induced models, mitochondrial dysfunction induced by TFAM deficiency results in energy crisis and progressive dopaminergic neuronal loss that is accompanied by the presence of intraneuronal cytoplasmic inclusions (albeit not α-synuclein-positive) (Sterky *et al.*, 2011). Supporting the relevance of these findings to the general human population, epidemiological studies revealed that chronic exposure to rotenone (which is popularly used as a pesticide in farming) renders individuals susceptible to PD (Tanner *et al.*, 2011). Similarly, regular consumption of fruit and tea from annonaceous plants such as soursop that contain annonacin, a complex I inhibitor, is linked to the development of atypical parkinsonism in French West Indies (Guadeloupe) (Caparros-Lefebvre and Elbaz, 1999). Taken together, a role of mitochondrial dysfunction in PD pathogenesis thus appears compelling.

How mitochondria become defective in the PD brain is less clear. As mentioned earlier, mitochondria are not solitary and static structures as depicted in many textbooks but rather

are dynamic and mobile organelles that constantly undergo membrane remodeling through repeated cycles of fusion and fission. In addition, the organelle also undergoes regulated turnover via a specialized form of autophagy known as "mitophagy" when it is damaged beyond repair. It follows that mitochondrial dysfunction can occur at different levels ranging from organelle biogenesis, fusion/fission to mitophagy. Indeed, genetic mutations that disrupt the function of mitochondrial fusion/fission regulators leads to neurodegenerative diseases such as Charcot-Marie-Tooth type 2A and autosomal dominant optic atrophy although not PD *per se* (for a recent review, refer to Chen and Chan, 2009). Recently, an attractive mechanism underlying mitochondrial dysfunction that involves failed mitophagy has emerged that might potentially explain how and why mitochondrial impairments occur in PD, at least for *PARK2*-related cases.

PARK2/PARKIN AND INTRACELLULAR QUALITY CONTROL

Mutations in the *PARK2* gene were originally identified in Japan more than a decade ago to be causative of autosomal recessive juvenile parkinsonism (Kitada *et al.*, 1998). Following this discovery, several ethnically diverse individuals with early-onset PD (age <45 years) in other parts of the world were also found to carry Parkin mutations, which occur at a frequency of about 10–20% and 50% in sporadic and familial early-onset cases, respectively. *PARK2* encodes Parkin, a RING-HECT hybrid E3 ubiquitin ligase whose function is normally associated with the ubiquitin-proteasome system (UPS), a major proteolytic system that identifies and degrades unwanted intracellular proteins (Figure 11.1). In this system, proteins that are destined for proteasome-mediated degradation usually have a chain of ubiquitin added via a reaction cascade that involves the ubiquitin-activating (E1), -conjugating (E2) and -ligating (E3) enzymes, whereby successive iso-peptide linkages are formed between the terminal residue (G76) of one ubiquitin molecule and a lysine (K) residue (most commonly K48) within another. The (G76-K48) polyubiquitinated substrate is then recognized by the 26S proteasome as a target for degradation. Because the ubiquitin sequence contains seven lysine residues (i.e., at positions 6, 11, 27, 29, 33, 48, and 63), the assembly of a polyubiquitin chain utilizing alternative lysine residues (such as K63) can also occur. In addition, proteins can also be monoubiquitinated. Notably, both K63-linked ubiquitination and monoubiquitination of proteins are not typically associated with proteasome-mediated degradation.

We and others have demonstrated that Parkin is a unique, multifunctional enzyme capable of mediating both proteasome-dependent K48-linked ubiquitination as well as alternative ubiquitin topologies such as monoubiquitination and K63-linked ubiquitination that are uncoupled from the proteasome (Lim *et al.*, 2006). Importantly, our collective results suggest that Parkin-mediated K63-linked ubiquitination promotes the perinucleus clustering of misfolded proteins and thereby their sequestration into aggresomes and subsequently primes them for clearance by macroautophagy (herein refer to as autophagy) (Tan *et al.*, 2009). By being capable of mediating both proteasome-associated K48-polyubiquitination and autophagy-associated K63-linked polyubiquitination, Parkin may potentially act as an important triage between the two major cellular degradation systems. This multifunctionality of Parkin may in part help to explain its apparent broad neuroprotective properties, as

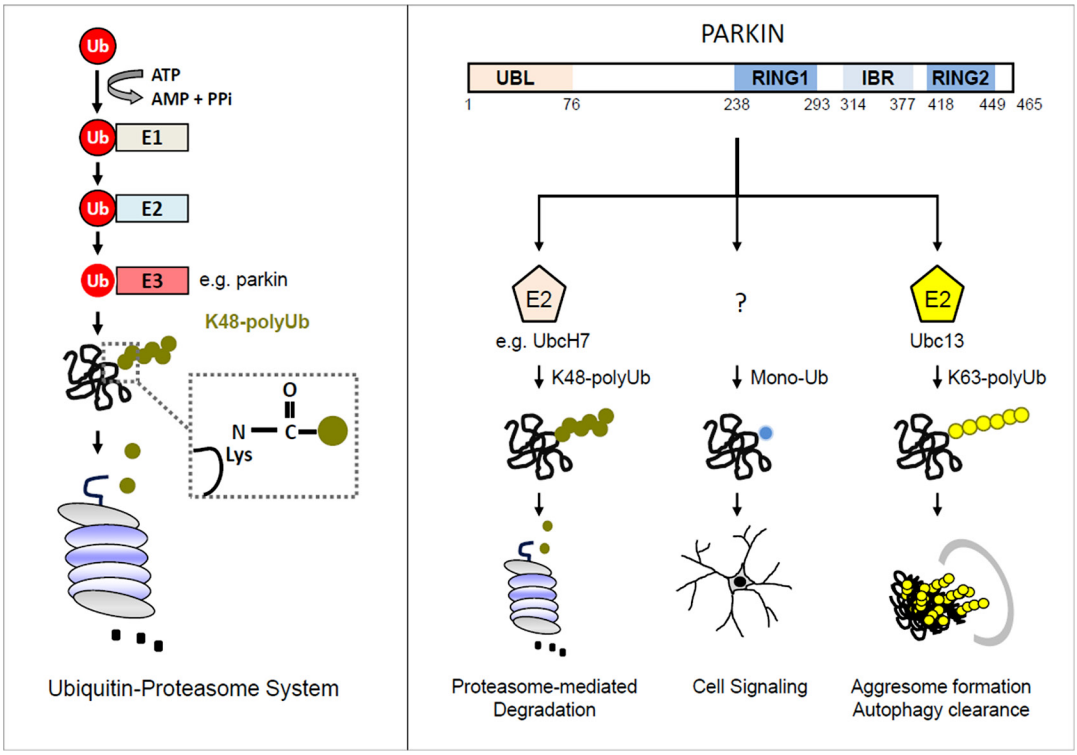

FIGURE 11.1 UPS and multifunctional catalytic properties of Parkin. (*Left*) Schematic depiction of the UPS showing that E1, E2, and E3 collaborate to assemble ubiquitin chains (K48-linked) on a target protein, which is then recognized by the proteasome for degradation. (*Right*) Parkin is a unique E3 capable of mediating ubiquitin modification of different topologies. Parkin interaction with UbcH7 promotes K48-linked polyubiquitination whereas its partnership with Ubc13 appears to favor K63-linked polyubiquitination. It is unclear whether an E2 is required for Parkin-mediated monoubiquitination to occur. Monoubiquitination of proteins is involved in cell signaling while K63-linked polyubiquitination may promote the formation of aggresomes and their subsequent clearance by autophagy.

the flexibility of ubiquitin linkage usage presumably would allow the enzyme to adapt rapidly to changes in the cellular environment.

One of the first hints that Parkin may play a role in mitochondrial homeostasis aside from its more obvious role as a regulator of protein turnover came from a study in fruit flies. Interestingly, adult *Drosophila Parkin* null mutant exhibits its most prominent pathology not in the brain but in the flight musculature, which is plagued by muscle degeneration and pronounced mitochondrial lesions (Greene *et al.*, 2003). Following this discovery, *PINK1*, a related PD-linked gene encoding a mitochondrial serine/threonine kinase, was similarly implicated through *Drosophila*-based studies as a regulator of mitochondrial homeostasis. *PINK1* null flies were found to phenocopy their Parkin-deficient counterparts and importantly, Parkin overexpression in PINK1$^{-/-}$ flies was able to rescue all the mutant phenotypes tested, although the reverse did not happen, suggesting that Parkin acts in the same

pathway but downstream of PINK1. We now know from several follow-up studies in flies and other model systems that the Parkin/PINK1 pathway is an important regulator of mitochondrial dynamics, although it is currently controversial whether the pathway promotes mitochondrial fission or fusion (for a recent review, refer to Guo, 2010).

In an exciting development, Narendra and colleagues from Youle laboratory have recently demonstrated that Parkin plays an essential role in removing damaged mitochondria from the cell via mitophagy (Narendra *et al.*, 2008). This seminal discovery has fueled widespread interest among many researchers directed at elucidating the mechanism underlying Parkin-mediated mitophagy that continues to this date. Accordingly, impairment in mitochondrial QC due to failed mitophagy in Parkin-deficient neurons has been proposed to be a key mechanism that predisposes them to degeneration. However, much of what we currently know about the mechanism underlying Parkin-mediated mitophagy is derived from studies with Parkin-overexpressing cells typically treated with nonphysiological concentrations of chemical uncouplers (e.g., 10 M of carbonyl cyanide m-chlorophenylhydrazone [CCCP]) to collapse the mitochondrial membrane potential ($\Delta\psi m$). Upon exposure to such concentrations of protonophores for 24–48 hours, Parkin-expressing (but not Parkin-deficient) cells tend to have complete or near complete loss of their mitochondria, as evidenced by immunostaining with mitochondrial markers such as TOM20 and by electron microscopy analysis. It remains intriguing how cells could survive without mitochondria for prolonged periods. Undeniably, this is of course a "sledgehammer" approach towards promoting mitochondrial depolarization and whether or not such en-bloc organelle damage and ensuing clearance are physiologically relevant or not is questionable, although we and others have shown that several disease-associated Parkin mutants failed to elicit mitophagy in cells treated with CCCP (which implies a certain degree of patho-physiological relevance) (Lee *et al.*, 2010a). Nonetheless, the above approach has turned out to be extremely popular among investigators in their quest to elucidate the mechanism underlying Parkin-mediated mitophagy. A model that has emerged from a flurry of such activities is depicted in Figure 11.2. Broadly (and somewhat arbitrarily), the whole mitophagy process may be divided into four interrelated steps, as follows:

Step 1 – *PINK1 stabilization on the mitochondrial outer membrane*. According to the proposed model, a key initial event that occurs upon mitochondrial depolarization is the selective accumulation of PINK1 on the outer membrane of the damaged organelle. This event does not occur in healthy mitochondria as the PINK1 protein containing a mitochondrial-targeting signal (MTS) at its N-terminus is normally imported into the inner mitochondrial membrane (IMM) through the sequential actions of the translocase of outer mitochondrial membrane (TOM) complex and the translocase of inner mitochondrial membrane (TIM) complex. During the importation process, the full-length 63 kDa PINK1 is progressively modified by a series of mitochondrial proteases. The first of these is the mitochondrial processing peptidase (MPP), which cleaves the MTS away from PINK1 to generate a 60 kDa form. This truncated form is then further processed by the presenilin-associated rhomboid-like protease (PARL) (and/or the m-AAA protease) to a 52 kDa protein, which is rapidly degraded by the proteasome (which presumably would require PINK-52 to be retro-translocated to the cytosol via the mitochondrial surface) or by an unknown mitochondrial protease that is sensitive to

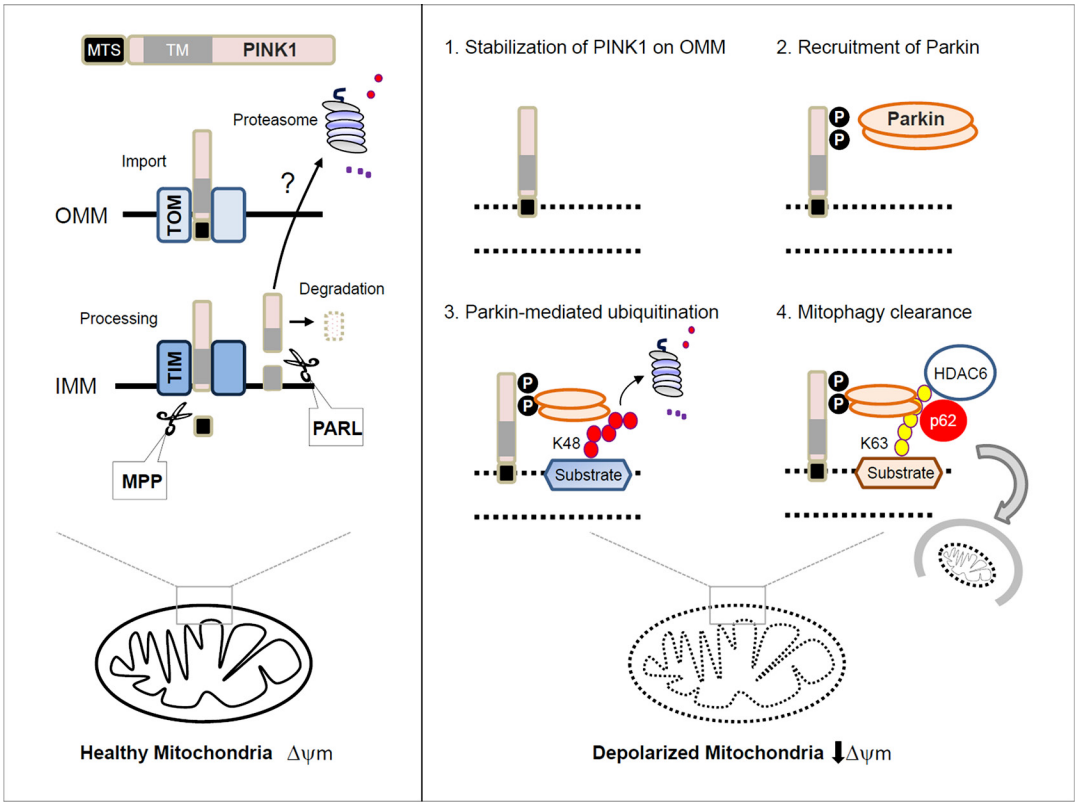

FIGURE 11.2 Model of Parkin/PINK1-mediated mitophagy. (*Left*) In healthy mitochondria, PINK1 imported through the outer mitochondrial membrane (OMM) is rapidly processed and degraded. (*Right*) Upon mitochondrial depolarization, PINK1 stabilization on the OMM leads to its dual autophosphorylation, which triggers Parkin recruitment, self-association, and catalytic activation. Parkin ubiquitinates several proteins on the OMM that results in their degradation by the proteasome recruited on the mitochondrial surface. Mitophagy induction then occurs presumably assisted by the autophagy adaptor proteins p62 and HDAC6 that bind to K63 polyubiquitinated proteins that are enriched on the damaged organelle (*see text for details*).

proteasome inhibitors (Greene *et al.*, 2012; Jin *et al.*, 2010). Although the above proteolytic events would ensure that PINK1 is kept at low levels under normal conditions (and as such to prevent mitophagy from occurring), it is intriguing at the same time to note that the cell has to go through such an elaborate process (i.e., to synthesize, import, cleave it twice, and degrade the protein) just to restrict its expression. It is attractive therefore to speculate that the 52 kDa PINK1 protein might be stabilized under certain conditions to subserve some cellular roles. Supporting this, accumulation of PINK 52, but not the full-length protein, has been reported in the brains of both idiopathic and PINK1-related PD patients, possibly reflecting a response to PD-linked stress (Muqit *et al.*, 2006). Notwithstanding this, under conditions of acute mitochondrial depolarization (typically achieved experimentally by the treatment of cells with mitochondrial uncouplers such

as CCCP or valinomycin), the importation of PINK1 (a Δψm-dependent process) is inhibited due to the dissipation of Δψm, which allows full-length PINK1 to accumulate on the outer mitochondrial membrane (OMM). Accumulated PINK1 apparently forms a complex with TOM on depolarized mitochondria, presumably to facilitate its reimport into a subset of repolarized mitochondria that are rescued from mitophagy (Lazarou *et al.*, 2012).

Step 2 – *Recruitment of Parkin by PINK1 on the mitochondrial outer membrane*. Upon its accumulation on the OMM of depolarized mitochondria, PINK1 undergoes autophosphorylation at Ser228 and Ser402, a post-translational modification that appears to be important for the recruitment of Parkin (Okatsu *et al.*, 2012). This event somehow triggers Parkin self-association and unmasks its latent ubiquitin ligase activity (Lazarou *et al.*, 2013). Interestingly, when PINK1 is experimentally made to localize to peroxisomes or lysosomes, it can also mediate the recruitment and concomitant catalytic activation of Parkin on these organelles (which results in autophagy-mediated clearance of peroxisomes but not lysosomes). In contrast, targeting Parkin alone to mitochondria, peroxisomes, or lysosomes in the absence of PINK1 fails to stimulate its activity (Lazarou *et al.*, 2012). Thus, membrane-localized PINK1 seems to be necessary for Parkin recruitment. In a reciprocal fashion, Parkin is phosphorylated at Ser65 in a PINK1-dependent manner upon depolarization of Δψm, although whether PINK1 phosphorylates Parkin directly or indirectly via the activation of another kinase remains unresolved (Shiba-Fukushima *et al.*, 2012). Parkin Ser65 resides within its UBL domain (which normally folds in a manner that would keep the ubiquitin ligase in a repressed state) and its phosphorylation is thought to relieve the autoinhibition of Parkin E3 activity (Kondapalli *et al.*, 2012) as well as promote the translocation of the protein to depolarized mitochondria (Shiba-Fukushima *et al.*, 2012). Notably, some studies suggest that besides PINK1, voltage-dependent anion channels (VDACs – OMM porin proteins) may also be involved in the recruitment of Parkin, although the mechanism underlying this is poorly understood (Geisler *et al.*, 2010).

Step 3 – *Ubiquitination of mitochondrial proteins by Parkin*. Once recruited onto the mitochondria, Parkin becomes activated and promotes the ubiquitination and subsequent degradation of many OMM proteins including the pro-fusion mitofusin proteins, the elimination of which is thought to prevent unintended fusion events involving damaged mitochondria and thereby their reentry into the undamaged mitochondrial network from occurring (Poole *et al.*, 2010; Ziviani *et al.*, 2010). Mitofusin degradation is regulated by p97 (an AAA-ATPase linked to ER-associated degradation), which accumulates on depolarized mitochondria to presumably facilitate the extraction of ubiquitinated mitofusins (and possibly other Parkin substrates) from the outer membrane for degradation by the proteasome (Tanaka *et al.*, 2010). Besides mitofusins, Parkin also mediates the degradation of several other OMM proteins such as Tom 20, Tom 40, Tom 70 and Omp 25 of depolarized mitochondria (Chan *et al.*, 2011; Yoshii *et al.*, 2011). To better define the repertoire of mitochondrial substrates that Parkin acts on, a recent study conducted by Sarraf and colleagues utilized quantitative diGLY proteomics to elucidate the ubiquitination site-specificity and topology of Parkin-dependent target modification in response to mitochondrial depolarization (Sarraf *et al.*, 2013). By means of this unbiased approach, they identified hundreds of ubiquitination sites that are

dynamically regulated and evolutionarily conserved in a large number of proteins, with strong enrichment of OMM proteins. Further, they found via complementary interaction proteomics that Parkin associates with these targets, as well as with autophagy receptors in a depolarization-dependent manner and that disease-associated C431 mutation of Parkin disrupts these associations. Whether p97-mediated extraction is needed here for Parkin to ubiquitinate the various OMM proteins is unclear, although it may seem cumbersome for the cell to do so given that a large number of mitochondrial proteins would have to be extracted. According to recent studies, Parkin may instead activate the UPS upon translocation to the mitochondria to facilitate the widespread degradation of mitochondrial proteins (Chan *et al.*, 2011). This involves the enrichment of K48-linked ubiquitination of OMM proteins and the recruitment of the proteasome to the mitochondria, which may lead to rupturing of the mitochondrial outer membrane (Chan *et al.*, 2011; Yoshii *et al.*, 2011). Despite the apparently massive degradation of OMM proteins associated with mitophagy, a recent report suggests that the phenomenon is a selective process in that certain OMM proteins such as FKBP38 and Bcl-2 can escape the degradation by translocating from the mitochondria to the ER during the process, which apparently is needed to keep apoptosis suppressed during mitophagy (Saita *et al.*, 2013). The number of basic amino acids residing within a short juxta-membrane sequence at the COOH-terminus of FKBP38 and Bcl-2 appears to be important for the translocation to occur. Moreover, it is also clear now that some targets of Parkin are enriched (rather than reduced) in response to mitochondrial depolarization (Chan *et al.*, 2011; Sarraf *et al.*, 2013), presumably as a result of Parkin-mediated K63-linked ubiquitination. Thus, mitophagy-associated degradation of proteins appears to be a selective process that discriminates degradation-associated targets from those that need to evade the destruction. Being a multifunctional ubiquitin ligase capable of mediating ubiquitin chains of different topologies, Parkin is especially well-suited for the job. However, the cues that determine Parkin's preference to mediate a particular type of ubiquitin chain formation remain elusive.

Step 4 – *Recruitment of autophagy apparatus for mitophagy*. Among the proteins that are enriched in response to mitochondrial depolarization is p62, an autophagy adaptor that is crucial for the perinucleus clustering of depolarized mitochondria (Okatsu *et al.*, 2010). The mitochondrial recruitment of p62 is thought to occur via its affinity for K63-linked chains, which proteins on the damaged organelle (particularly those that are not destined for proteasome-linked degradation) are also decorated with. However, whether p62 plays an essential role in directing mitophagy is currently contentious, although the proposal is attractive given that p62 also interacts with LC3 (an autophagosome marker) and may as such play a role in autophagosome recruitment during mitophagy. Whereas we and others have provided evidence to support such a model (Geisler *et al.*, 2010; Lee *et al.*, 2010a), some other groups have found that p62 is not required for mitophagy (Ding *et al.*, 2010; Okatsu *et al.*, 2010). Alternatively, another ubiquitin-binding protein known as HDAC6 may be involved. Like p62, HDAC6 also preferentially binds K63-linked chains. By virtue of its ability to bind to both K63-linked ubiquitinated proteins and dynein motors, HDAC6 may facilitate the dynein-dependent transport of depolarized mitochondria via the microtubule network to the juxtanuclear region. Indeed, we have shown that HDAC6 translocates to damaged mitochondria in a Parkin-dependent

manner and that mitochondrial clustering and subsequent mitophagy fail to take place in the absence of HDAC6 expression (Lee *et al.*, 2010a). Notably, HDAC6 has been shown to stimulate autophagosome-lysosome fusion via the recruitment of a cortactin-dependent, actin remodeling machinery (Lee *et al.*, 2010b). Not surprisingly, cortactin expression silencing similarly results in mitophagy failure (Lee *et al.*, 2010a). Taken together, it is attractive to suggest that p62 and HDAC6 may work independently or in concert to promote the final removal of damaged mitochondria by the lysosome.

Notwithstanding the logic of the above model, a recent study from Mizushima's lab revealed that the initial cargo recognition step of mitophagy does not involve the interaction between LC3 and the adaptor molecules. Rather, Parkin recruitment on the mitochondria induces the formation of ULK1 (Atg1) puncta and Atg9 structures (Itakura *et al.*, 2012). Because the ULK1 complex functions as an essential upstream nucleation step of the hierarchical autophagy cascade, their results suggest that the mitophagosome is generated in a *de novo* fashion on damaged mitochondria. Autophagosomal LC3 is, however, important for the efficient incorporation of damaged mitochondria into the autophagosome at a later stage. Conceivably, both de novo and pre-existing autophagosomes may be involved in mitophagy, the extent of each perhaps depending on the availability of selected membranes. Nonetheless, how Parkin participates in the *de novo* synthesis of isolation membrane was not clarified, although an interesting report by Van Humbeeck and colleagues suggests that Ambra1 (activating molecule in Beclin 1-regulated autophagy), an autophagy-promoting protein, may be involved (Van Humbeeck *et al.*, 2011). Ambra1 is known to induce autophagy by stimulating the activity of class III phosphatidylinositol 3-kinase (PI3K) complex (comprising Vps34, Beclin 1 and p150), which is essential for the formation of new phagophores (Fimia *et al.*, 2007). Importantly, Ambra1 interacts with Parkin and their association is enhanced following prolonged mitochondrial depolarization (Van Humbeeck *et al.*, 2011). Although the autophagy-promoting protein is not required for Parkin translocation or Parkin-mediated perinucleus aggregation of depolarized mitochondria, it is critical for the subsequent clearance of the organelle (Van Humbeeck *et al.*, 2011). Interestingly, Ambra1 is phosphorylated by ULK1 during starvation-induced autophagy, which suggests the attractive possibility that Parkin, ULK1, and Ambra1 may be linked together in the same mitophagy-associated pathway.

RELEVANCE OF PARKIN-MEDIATED MITOPHAGY TO PARKINSON'S DISEASE PATHOGENESIS

Although the model described above is for the most part logical and elegant, it is not without controversy. A recurrent and important question among PD researchers regarding the proposed model is whether mitophagy induced by Parkin in dividing cells treated with mitochondrial poisons is at all relevant to postmitotic neurons, particularly dopaminergic neurons associated with PD. Further, unlike cultured cells that generate most of their ATP via glycolysis from glucose typically present in the medium, the bioenergetics of neurons are strictly dependent on mitochondrial respiration and may as such elicit a different response towards mitochondrial depolarization. According to a recent study by Van Laar

and colleagues (Van Laar *et al.*, 2011), Parkin's translocation to depolarized mitochondria is indeed attenuated in cultured cells forced into dependence on mitochondrial respiration (i.e., cultured in glucose-free medium). Similarly, CCCP treatment of primary neurons neither promotes the translocation of Parkin (both exogenous and endogenous) to the mitochondria nor triggers mitophagy. More recently, using primary human fibroblasts and induced pluripotent stem cell (iPS)-derived neurons from controls and PINK1 mutation carriers as models, Rakovic and colleagues demonstrated that endogenous Parkin is insufficient to initiate mitophagy in these models regardless of the functional status of PINK1. They also found that Parkin overexpression can "rescue" the "defective mitophagy" only in fibroblasts but not in iPS-derived neurons (Rakovic *et al.*, 2013). However, their finding is in conflict with an earlier report by Seibler and colleagues, who showed that the impairment of Parkin recruitment to depolarized mitochondria in iPS-derived dopaminergic neurons from PINK1-related PD patients can be rescued by reintroduction of wild-type PINK1 into PINK1-deficient neurons (Seibler *et al.*, 2011). Moreover, at least two other groups have found that Parkin accumulation on mitochondria does take place in primary neurons treated with CCCP (Narendra *et al.*, 2008; Vives-Bauza *et al.*, 2010). Furthermore, using *Drosophila* as a model, a very recent study provided evidence that Parkin-mediated mitophagy occurs *in vivo* (Vincow *et al.*, 2013). The investigators found that the brains of *Parkin* mutant flies exhibit a significantly decreased rate of mitochondrial protein turnover, which is similar to that produced by general autophagy blockade induced by the genetic ablation of *atg7*. Their results suggest that Parkin indeed promotes mitochondrial turnover through autophagy and that the process is physiologically relevant, albeit in the fly. Corroborating these findings, we have recently reported that AMP kinase (AMPK), a key cellular energy regulator that is known to activate autophagy, can restore mitochondrial homeostasis that is otherwise compromised in Parkin null flies (Ng *et al.*, 2012). Not surprisingly, we found that AMPK activation could ameliorate all the reported pathological phenotypes of Parkin null flies, including dopaminergic neurodegeneration and associated locomotion deficits. In a related development, Haskin and colleagues have recently uncovered AF6 (an F-actin binding protein) as a novel interactor of Parkin and demonstrated a previously unreported role for AF6 in mitophagy in that AF6 could augment the Parkin/PINK1 pathway to promote the removal of damaged mitochondria via mitophagy (Haskin *et al.*, 2013). The study highlighted that endogenous Parkin may require additional factors (that may be rate-limiting in neurons) to orchestrate mitophagy efficiently. Taken together, the verdict remains open regarding the validity of the proposed Parkin/PINK1 mitophagy model in the neuronal context.

Notwithstanding the preceding, even if Parkin-mediated mitophagy is indeed taking place in neurons, a pertinent question to ask is whether deficient mitochondrial QC is relevant at all to the large number of sporadic PD cases where Parkin is not mutated. Although this remains to be established, it is noteworthy to mention that we and others have previously shown that Parkin dysfunction could arise in the PD brain in the absence of apparent mutations, that is through modifications of the wild type protein leading to its functional impairments. This could be a result of stress-induced biochemical alterations including oxidation and nitrosylation, post-translational modifications, or aberrant protein-protein interaction that can either alter the catalytic function of the E3 ligase directly, or indirectly through promoting its aggregation or degradation (Tan *et al.*, 2009). Interestingly, normal

Parkin in the brain also becomes progressively more detergent-insoluble (and therefore non-functional) with aging (Pawlyk *et al.*, 2003), which may provide an explanation of why age represents a risk factor for PD. In all these cases, the loss of Parkin function is expected to compromise the efficiency of Parkin-mediated mitophagy, among other Parkin-regulated events. Thus, deficient mitochondrial QC may not necessarily be restricted to cases where Parkin (or PINK1) is overtly mutated. Consistent with this, AF-6 (which augments Parkin-mediated mitophagy) was found to be present in LBs and its soluble levels are strikingly decreased in the SN and striatum of sporadic PD patients, suggesting that reduced availability of AF-6 may compromise the efficiency of Parkin-mediated mitophagy and contribute to the accumulation of dysfunctional mitochondria in the disease (Haskin *et al.*, 2013). Finally, it is also becoming increasingly clear that various other PD-linked proteins that may appear to have disparate functions could all interact with the Parkin/PINK1 pathway to influence mitochondrial QC, either directly or indirectly.

CONCLUDING REMARKS

Although a flurry of activity (i.e., after the original report by the Youle lab) to elucidate the precise mechanism underlying Parkin-mediated mitophagy ensues to this date, the model (like all newly proposed models) remains imperfect and will continue to evolve. The main caveat is that the proposed Parkin/PINK1 pathway represents a cellular response to a sudden, catastrophic dissipation of $\Delta\psi m$ that quite obviously does not mirror the gradual, age-dependent decline in mitochondrial function occurring in the PD brain. Notably, the disease usually takes decades to manifest even in individuals with overt *PARK2* mutations. Paradoxically, the controversies that the model has generated may serve as an impetus for labs around the world in directing efforts towards clarifying the precise role of Parkin-mediated mitophagy in PD pathogenesis, which would be a good thing to happen. However, because mitophagy and autophagy are essentially two peas in the same pod, it remains a huge challenge to demonstrate unequivocally that mitophagy impairment, instead of a generalized impairment in the autophagy process, contributes directly to neurodegeneration *in vivo*. This would require the genetic differentiation of targeted components that are exclusively involved in mitophagy. Currently, key components of mitophagy and autophagy tend to overlap and it does not help when Parkin appears to subserve both types of autophagy processes. Thus, although mitochondrial QC is invariably important for neuronal survival, whether failure in the removal of damaged mitochondria is in itself a driver of disease pathogenesis or is a consequence of a progressive and general decline in autophagy function in the PD brain will be a convoluted puzzle to unravel. Nonetheless, thanks to the proposed Parkin/PINK mitophagy model, we now have an important jigsaw piece to fit on to the puzzle board.

Acknowledgments

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CHAPTER

12

Ubiquitination of Mitofusins in PINK1/Parkin-Mediated Mitophagy

Matthew E. Gegg

OUTLINE

Abstract

The maintenance of a healthy pool of mitochondria is vital for cellular physiology and survival. Quality control mechanisms exist to ensure that oxidative phosphorylation is maintained and the production of highly damaging reactive oxidizing species by damaged mitochondria is minimized. This is thought to be particularly important for neurons, which have high energy requirements and cannot dilute the burden of dysfunctional mitochondria by cell division. The targeted removal of damaged mitochondria is achieved by macroautophagy, which is also known as mitophagy. Mitochondrial and lysosomal function decrease with age and therefore both are implicated in aging and age-related disorders such as Parkinson's disease. The discovery that PINK1 and Parkin, two proteins associated with familial forms of Parkinson's disease, are involved in mitophagy has strengthened this hypothesis. PINK1 and Parkin identify damaged mitochondria for degradation by mitophagy via the ubiquitination of several mitochondrial proteins. Two such proteins are mitofusins 1 and 2, whose normal function is to mediate the fusion of mitochondria. This chapter describes the process by which PINK1 and Parkin identify damaged mitochondria and discuss the putative mechanism(s) by which ubiquitination of the mitofusins facilitates mitophagy.

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INTRODUCTION

Mitochondria are fundamental to the health and survival of cells, generating ATP via oxidative phosphorylation, playing a role in calcium homeostasis, and modulating apoptosis. A byproduct of oxidative phosphorylation is the generation of reactive oxygen species (ROS) by the electron transport chain (ETC) that can damage protein, lipids and DNA, not only of mitochondria themselves, but other components of the cell. Dysfunctional mitochondria, and, in particular, impairments to the ETC, will generate greater levels of ROS. Therefore it is imperative that old or damaged mitochondria are efficiently degraded by macroautophagy. This process has been termed mitophagy.

Mitochondrial and lysosomal function decrease with age, and both have been implicated in neurodegenerative disorders such as Parkinson's disease (PD) and Alzheimer's disease (reviewed in Nixon, 2013; Schapira, 2008; Terman *et al*., 2010). Neurons are particularly susceptible to decreased quality control mechanisms for mitochondria as they are high energy users, with as much as 20% of the body's oxidative phosphorylation occurring in the central nervous system (Van Laar and Berman, 2013). Neurons are also postmitotic, and therefore cannot dilute out damaged mitochondria by mitosis.

Mutations in the *PINK1* and *parkin* genes are a cause of autosomal recessive PD. Loss of function of either of these proteins has been known for several years to cause mitochondrial dysfunction (reviewed in Schapira and Gegg, 2011; Van Laar and Berman, 2013). However, it was the identification that PINK1 and Parkin act in the same pathway (Park *et al*., 2006) and their involvement in mediating mitophagy (Matsuda *et al*., 2010; Narendra *et al*., 2008; Vives-Bauza *et al*., 2010) that have led to a putative explanation for the mitochondrial dysfunction seen in these rare familial forms of PD (Gegg *et al*., 2010), but also perhaps sporadic forms of PD and other disorders (Dorn, 2013).

In this chapter will be briefly reviewed the fundamentals of mitochondrial dynamics, as they are intrinsic to the mitophagy process. Then will be described how PINK1 and Parkin identify damaged mitochondria for elimination by mitophagy, with particular focus on the role ubiquitination of mitofusins plays in this.

MITOCHONDRIAL DYNAMICS

Analysis of mitochondria in living cells has shown that these double-membrane-bound organelles are not static but undergo fusion and fission events with each other (Figure 12.1). The dynamics of these mitochondrial networks changes depending on cellular metabolism requirements, specific cellular functions, and mitochondrial/cellular stress (reviewed by Youle and van der Bliek, 2012; Zorzano *et al*., 2010).

Mitochondria undergo fission when they are needed to move to a different location within the cell. For example, fission occurs during mitosis so that daughter cells are populated with mitochondria. Transport of mitochondria in neurons occurs over relatively large distances, where mitochondria are transported from the soma to synaptic terminals, and vice versa (Cai *et al*., 2012; Wang *et al*., 2011). Mitochondria also fragment prior to apoptosis or when they are identified for degradation by mitophagy (Dorn, 2013; Twig *et al*., 2008; Van Laar and Berman, 2013).

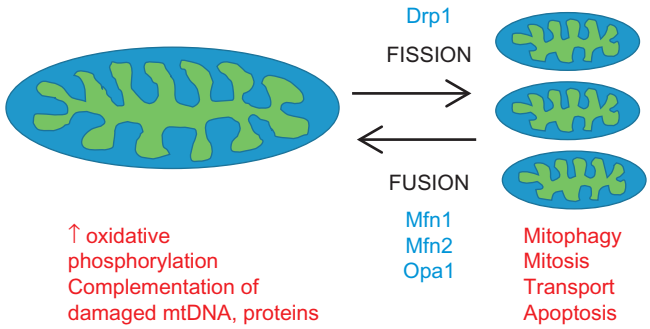

FIGURE 12.1 Mitochondria are dynamic organelles that can undergo both fission and fusion in order to meet the physiological requirements of the cell. The fusion of mitochondria is mediated principally via Mfn1, Mfn2, and Opa1, while Drp1 protein is an important inducer of mitochondrial fission.

Conversely, mitochondria fuse in order to maximize oxidative phosphorylation when energy demand is high, but it is also a mechanism to reduce the impact of damage to mitochondrial DNA (mtDNA) and proteins of the ETC. Mutations and deletions arise in mtDNA, which encodes for several proteins required for oxidative phosphorylation (Bender *et al.*, 2006; Terman *et al.*, 2010). Two significant contributors to mtDNA damage are mitochondrial ROS production, and less sophisticated DNA repair mechanisms in mitochondria. Fusion of mitochondria will result in a heteroplasmic mix of wild-type and mutant mtDNA, thus allowing normal mitochondria to compensate for deficiencies of damaged mitochondria up to a particular threshold, whereupon damaged mitochondria would be removed by mitophagy (Dorn, 2013; Van Laar and Berman, 2013; Zorzano *et al.*, 2010).

The fission and fusion of mitochondria are mediated by several proteins. Fission in mammalian cells is principally mediated via the dynamin family member Drp1. Drp1 is recruited to mitochondria from the cytosol, which then forms spirals around mitochondria, thus constricting both the outer and inner mitochondrial membranes. Fusion of mitochondria is principally controlled by three proteins: mitofusin 1 and 2 (Mfn1, Mfn2) and OPA1. The fusion of mitochondrial outer membranes (MOMs) is controlled by Mfn1 and Mfn2, while OPA1 resides at the mitochondrial inner membrane (MIM), and mediates the fusion of this membrane (Hoppins *et al.*, 2011; Youle and van der Bliek, 2012; Zorzano *et al.*, 2010). Since this chapter concerns Mfn1 and Mfn2, these two proteins will be briefly focused on.

Mfn1 and Mfn2 are both transmembrane GTPases with 63% identity and share the same functional domains (five G motifs in the GTPase domain and two coiled coil domains; Zorzano *et al.*, 2010). Mfn1 and Mfn2 form both homotypic and heterotypic complexes between two separate membranes to fuse them, with Mfn1-Mfn2 heterotypic dimers recently suggested in an *in vitro* fusion assay to have greater efficacy (Hoppins *et al.*, 2011). Mfn2 also has a proline rich domain for further protein-protein interactions, and this might explain the additional functions attributed to Mfn2. Ablation of either Mfn1 or Mfn2 is embryonic-lethal due to placental defects. Conditional knockout of Mfn1 in mice, where expression of Mfn1 is only maintained in the placenta, results in viable mice, with presumably any fusion defects compensated for by Mfn2. However, similar conditional Mfn2 knockout mice exhibit movement defects and impaired cerebellar development (Dorn, 2013;

Zorzano *et al*., 2010). Furthermore, autosomal dominant mutations in the *Mfn2* gene cause the neurodegenerative disorder Charcot-Marie-Tooth 2A disease. Finally, although both Mfn1 and Mfn2 are ubiquitously expressed, Mfn2 expression is greater in skeletal muscle, heart and brain, perhaps further explaining why loss of Mfn2 expression in the brain is more deleterious (Hoppins *et al*., 2011; Youle and van der Bliek, 2012; Zorzano *et al*., 2010).

PINK1/Parkin-MEDIATED MITOPHAGY

The mitochondrial membrane potential (ψm) is generated by the ETC chain on the MIM. It is the dissipation of this potential across the membrane that is used to drive the phosphorylation of ADP to ATP by ATP synthase. Impairment of the ETC or damage to mitochondria by other means typically results in a significant decrease in ψm. Mitochondria in such a state are described as being depolarized. A seminal study by Twig *et al*. (2008) demonstrated that it is depolarized mitochondria that are targeted for mitophagy, and that they are isolated from healthy mitochondria by undergoing fission from the mitochondrial network with the aid of Drp1.

The use of the mitochondrial uncoupling agent carbonyl cyanide m-chlorophenylhydrazone (CCCP) on predominantly proliferating mammalian cells has been instrumental in dissecting PINK1/Parkin-mediated mitophagy (Figure 12.2). Following depolarization of mitochondria and their fission from the network, the serine threonine kinase PINK1 accumulates on the MOM (Matsuda *et al*., 2010; Narendra *et al*., 2010; Vives-Bauza *et al*., 2010). Under normal conditions full length PINK1 (FL-PINK1) is imported into mitochondria and then the N-terminal containing the mitochondrial targeting sequence is cleaved by proteases at the MIM. The function of the processed form of PINK1 is still unclear, but it would appear to be rapidly turned over by the proteasome (Narendra *et al*., 2010). The accumulation of PINK1 on the MOM then recruits the E3 ubiquitin ligase Parkin from the cytosol to the mitochondria. The recruitment of Parkin to mitochondria occurs within 1 hour of depolarization by CCCP (Narendra *et al*., 2008). Parkin localization to depolarized mitochondria was abolished in MEFS from PINK1 knockout mice, but could be rescued by expressing exogenous WT-PINK1, but not kinase-dead PINK1 (Matsuda *et al*., 2010; Narendra *et al*., 2010). Therefore the recruitment of Parkin to depolarized mitochondria is dependent on the kinase activity of PINK1, although the precise mechanism is undefined. *In vitro* assays have indicated that Parkin can be directly phosphorylated by PINK1 (Kondapalli *et al*., 2012). Mutations in the *Fbxo7* gene are also a cause of autosomal recessive PD, and it has been shown that Fbxo7 protein binds to Parkin and is involved in the PINK1-dependent recruitment of Parkin to depolarized mitochondria (Burchell *et al*., 2013).

Regardless of the mechanism, once Parkin is recruited to mitochondria, there is an increase in the ubiquitination of mitochondrial resident proteins as assessed by immunofluorescence or western blotting (Gegg *et al*., 2010; Geisler *et al*., 2010; Matsuda *et al*., 2010). The first identified mitochondrial protein to be ubiquitinated by Parkin was VDAC1 (Geisler *et al*., 2010), which was then rapidly followed by the Mfns, first in *Drosophila* (Ziviani *et al*., 2010), and then the human SH-SY5Y neuroblastoma and Hela cell lines (Gegg *et al*., 2010, Tanaka *et al*., 2010). Mfn ubiquitination and the potential consequences of this will be discussed in the next section. A comprehensive proteomic approach in Hela, SH-SY5Y, and HCT116 cell lines confirmed the ubiquitination of MOM proteins such as VDAC1, Mfn1,

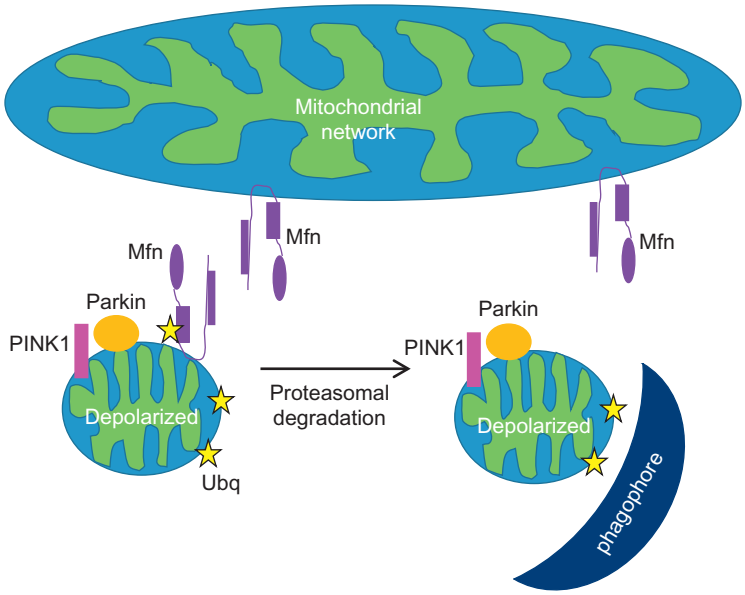

FIGURE 12.2 Depolarized mitochondria are segregated from the mitochondria network via fission. Loss of ψm causes PINK1 to accumulate at the mitochondrial outer membrane (MOM), whereupon it recruits parkin via a kinase-dependent mechanism. Parkin then ubiquitinates (Ubq) a number of MOM resident proteins including Mfn1 and Mfn2. Ubiquitination of Mfn1 and Mfn2 results in the degradation of these proteins by the proteasome. It has been proposed that this is a mechanism by which damaged mitochondria remain segregated, as they are unable to re-fuse with the mitochondrial network. Instead, damaged mitochondria are recruited to phagophores via other ubiquitinated proteins on the MOM for their destruction by mitophagy.

and Mfn2, but also identified hundreds of ubiquitination events on dozens of proteins following mitochondrial depolarization by CCCP (Sarraf *et al.*, 2013). While the majority of these proteins were located to the MOM, a number of cytosolic proteins, proteasome subunits, and autophagic receptors (SQSMT1/p62, CALCOCO2, TAX1BP1) were also identified. The authors noted that while these ubiquitination events were Parkin-dependent, they could not rule out the possibility that some ubiquitination events occur downstream of Parkin by other E3 ubiquitin ligases.

Following Parkin recruitment to mitochondria and ubiquitination, mitochondria are removed by mitophagy. Depolarized mitochondria co-localize with the autophagic proteins LC3 and p62 and have also been shown by electron microscopy to be engulfed by characteristic double membrane autophagosomes (Geisler *et al.*, 2010; Vives-Bauza *et al.*, 2010). The mitochondrial content of mammalian or *Drosophila* cells 24 hours after depolarization was significantly decreased when measured by immunofluorescence, western blotting, or mitochondrial enzyme activity (Gegg *et al.*, 2010; Narendra *et al.*, 2008; Ziviani *et al.*, 2010) consistent with the notion that mitochondria were being removed by mitophagy. Furthermore, mitochondrial loss was prevented in cells deficient for PINK1, Parkin, or ATG5, or when autophagy was inhibited by bafilomycin A1 or 3-methyladenine (Gegg *et al.*, 2010; Narendra *et al.*, 2008, 2010; Ziviani *et al.*, 2010).

Another MOM protein that is affected by Parkin-mediated ubiquitination is Miro, also known as RhoT (Sarraf *et al.*, 2013; Wang *et al.*, 2011). Miro is part of the motor/adaptor complex that is involved in mediating axonal transport of mitochondria (Wang *et al.*, 2011). PINK1/Parkin-dependent ubiquitination of Miro results in the degradation of Miro by the proteasome, resulting in the detachment of the kinesin motor from mitochondria, and thus arresting mitochondrial motility. It is likely that this prevention of movement serves to quarantine damaged mitochondria, allowing them to become engulfed by autophagosomes, whereupon they are returned to the soma for degradation by lysosomes (Cai *et al.*, 2012; Wang *et al.*, 2011).

Ubiquitination of Mfn1 and Mfn2 by Parkin

The first evidence that the Mfns were substrates of the PINK1/Parkin mitophagy pathway came from studies of the *Drosophila* homologue *dMfn* (also known as *Marf*). Under basal conditions, *PINK1* or *parkin* null mutant flies both had increased dMfn abundance, but not other mitochondrial proteins (Poole *et al.*, 2010; Ziviani *et al.*, 2010). In cultured S2R+ *Drosophila* cells, when dMfn was coexpressed with hemagglutinin-tagged ubiquitin, a number of higher molecular weight isoforms of dMfn, in addition to the expected 91 kDa full-length form, were detected (Ziviani *et al.*, 2010). These higher molecular weight forms were diminished when cells were treated with PINK1 or Parkin RNAi. Furthermore, immunoprecipitation assays indicated that Parkin could be detected in dMfn immunoprecipitates, and vice versa (Poole *et al.*, 2010; Ziviani *et al.*, 2010).

The polyubiquitination of endogenous Mfn1 and Mfn2 following depolarization of mitochondria was then found to occur in SH-SY5Y cells by western blot analysis. This Mfn ubiquitination was diminished in cells with PINK1 or Parkin RNAi (Gegg *et al.*, 2010). The ubiquitination of Mfn1 and Mfn2 was then reported in CCCP-treated Hela cells expressing exogenous Parkin (Chan *et al.*, 2011; Tanaka *et al.*, 2010). The membrane potential uncoupler valinomycin also induced Mfn1 and Mfn2 ubiquitination in human dermal fibroblasts, but was impaired in fibroblasts from patients with homozygous *PINK1* or *Parkin* mutations (Rakovic *et al.*, 2011). Finally a cell-free assay has shown that Parkin directly mediates the polyubiquitination of Mfn1 (Lazarou *et al.*, 2013). Note that no ubiquitination of the mitochondrial fission protein Drp1 or the fusion protein Opa1 has been detected following mitochondrial depolarization in either human or *Drosophila* models (Chan *et al.*, 2011; Gegg *et al.*, 2010; Rakovic *et al.*, 2011; Sarraf *et al.*, 2013; Tanaka *et al.*, 2010; Ziviani *et al.*, 2010).

The polyubiquitination of Mfn1 and Mfn2 occurs within 1 hour of mitochondrial depolarization and is rapidly followed by a decrease in Mfn protein levels leading to the premise that the Mfns are degraded by the proteasome (Chan *et al.*, 2011; Gegg *et al.*, 2010; Rakovic *et al.*, 2011; Tanaka *et al.*, 2010). The nature of the Mfn polyubiquitination is unclear. Parkin has been shown to mediate K27, K48, and K63-linked polyubiquitination. While K48-linked polyubiquitination is associated with degradation by the proteasome, K63 modifications are generally involved in autophagic clearance (Chan *et al.*, 2011). One cell free assay has suggested that Mfn1 undergoes both K48 and K63 polyubiquitination (Lazarou *et al.*, 2013). However, in SH-SY5Y cells, polyubiquitination of Mfn1 by Parkin has been reported to be atypical and independent of K48 and K63 (Glauser *et al.*, 2011). Proteomic analysis of Mfn1 has indicated that there are eight sites of polyubiquitination in Mfn1, two of which are

conserved in dMfn (Sarraf *et al*., 2013). All but two of the mammalian sites are located in helical motifs either side of the two C-terminal membrane spanning regions. All of the sites are cytosolic facing, consistent with the fact that Parkin is recruited to the outer leaflet of the MOM. Mfn2 also has multiple ubiquitination sites facing the cytosol either side of the transmembrane domains (Sarraf *et al*., 2013).

Despite the uncertain nature of the ubiquitination, inhibition of the proteasome with MG132 or epoxomicin prevented the rapid degradation of both Mfn1 and Mfn2 (Chan *et al*., 2011; Rakovic *et al*., 2011; Tanaka *et al*., 2010). The removal of Mfn1 and Mfn2 from mitochondria and their degradation by the proteasome appears to be dependent on p97/VCP, an AAA+ ATPase normally involved in the retro-translocation of ER proteins identified for proteasomal degradation (Tanaka *et al*., 2010). The kinetics of Mfn1 and Mfn2 polyubiquitination and their subsequent degradation by the proteasome appear to be the same in cultured cells (Chan *et al*., 2011; Gegg *et al*., 2010; Tanaka *et al*., 2010), although more classical pulse-chase experiments are required to confirm this. It should also be noted that expression of human Fbxo7 in *Drosophila* lacking Parkin decreased dMfn protein levels back to steady-state, suggesting that other ubiquitin ligases can also ubiquitinate Mfns during mitophagy, at least in *Drosophila* (Burchell *et al*., 2013).

All of the studies described have involved nonneuronal cell models. The post-mitotic nature of neurons and their well-documented reliance on oxidative phosphorylation for their energy needs should in theory mean that mitophagy is critical for the correct functioning and survival of neurons (Van Laar and Berman, 2013). However, the recruitment of Parkin to depolarized mitochondria in cortical neurons treated with CCCP is much slower than in proliferating cells. Parkin recruitment to mitochondria is extremely rare up to 6 hours after CCCP treatment, occasionally observed after 12 hours, and increasingly seen at 18 hours (Cai *et al*., 2012; Van Laar *et al*., 2011). Parkin-localized mitochondria were found to accumulate in the somatodendritic regions of neurons, where mature lysosomes are localized, and eliminated by autophagy. Notably, Parkin-localized mitochondria were hardly detected in axons or distal dendrites in this study, although LC3 co-localized mitochondria were (Cai *et al*., 2012). This may imply that (i) mitochondria recruit Parkin at undetectable levels in these two regions, (ii) Parkin rapidly localizes to mitochondria and then retranslocates back to the cytosol, or (iii) Parkin-independent mitophagy also occurs. To the author's knowledge, only one report has shown ubiquitination of Mfn1 and Mfn2 in primary neurons treated with CCCP. Weak ubiquitination could be seen 3 hours after CCCP treatment, which was diminished in neurons from Parkin knockout mice, or enhanced by expressing exogenous Parkin by lentiviral infection (Koyano *et al*., 2013). It was also notable that the authors who have detected PINK1/Parkin mediated mitophagy in neurons required very particular culturing conditions (Cai *et al*., 2012; Koyano *et al*., 2013). Given the findings of Cai *et al*. (2012), Mfn ubiquitination might be more robust at later time points.

CONSEQUENCES OF Mfn UBIQUITINATION

While there is little doubt that both Mfn1 and Mfn2 are ubiquitinated by Parkin following depolarization of mitochondria in proliferating cells, the specific effect(s) this has on mitophagy are still open for debate.

An attractive hypothesis is that the targeted degradation of Mfn1 and Mfn2 is a way of ensuring that damaged mitochondria that have undergone fission from the mitochondrial network, and recruited Parkin, remain segregated by preventing their re-fusion with healthy mitochondria, and are instead rapidly recruited to autophagosomes for elimination (Figure 12.2). A study using Hela cells (which have little or no endogenous Parkin) and Hela cells expressing YFP-parkin does support this idea (Tanaka *et al.*, 2010). Both cell types were exposed to CCCP for 90 minutes, resulting in the mitochondrial network becoming fragmented, and in the case of the Hela cells with YFP-parkin, mitochondria recruiting Parkin and becoming Mfn-deficient. The CCCP was then washed away and the recovery of the tubular mitochondrial network was analyzed over a 4-hour period by immunofluorescence. The recovery was slower in the Hela cells with YFP-parkin and Mfn-deficient mitochondria. A similar result was obtained using a fluorescence recovery after photobleaching (FRAP) assay. This time Hela cells with mitochondrial matrix targeted YFP (mitoYFP) expressing mCherry or mCherry-parkin were treated with CCCP for 90 mins. A region of interest was then photobleached, the CCCP washed away, and the rate of fluorescence recovery in the region of interest measured. Once again the cells without Parkin (and thus containing mitochondria with Mfns after CCCP treatment) showed a greater rate of recovery. These data suggest that Parkin-mediated degradation of Mfns promotes mitochondrial fragmentation after mitochondria become depolarized.

The large number of MOM proteins that are ubiquitinated upon Parkin localization to mitochondria could be used as an argument that Mfn ubiquitination is simply nonspecific and can be used merely as a "tag" for autophagic proteins such as LC3-II and p62 to recruit mitochondria to autophagosomes (Geisler *et al.*, 2010). Indeed, a study using a regulated heterodimerization system to target ectopic PINK1 to the outer membrane of mitochondria, peroxisomes, or lysosomes, indicated that Parkin was recruited to the respective organelles and mediated the ubiquitination of the resident membrane proteins (Lazarou *et al.*, 2012). In the case of mitochondria and peroxisomes this led to mitophagy and pexophagy, respectively.

The rate of mitophagy in WT MEFS and MEFS lacking both Mfn1 and Mfn2 was found to be similar in one report (Chan *et al.*, 2011), perhaps suggesting that ubiquitination of the Mfns is not critical for mitophagy progression, although more studies need to be performed. The limited and conflicting reports about the type of polyubiquitination Mfns are subjected to also make the situation far from clear. It is conceivable that the Mfns can simultaneously undergo different types of ubiquitination, fulfilling different functions.

However, the observation that some, if not all, of Mfn1 and Mfn2 is degraded by the proteasome implies that the ubiquitination of the Mfns serves a specific purpose. Indeed, the degradation of Mfn1 and Mfn2 by the proteasome would appear to be quicker than for other MOM proteins ubiquitinated by Parkin such as TOM20 and VDAC1, as assessed by western blotting (Chan *et al.*, 2011; unpublished observations). Immunostaining of damaged mitochondrial tubules in Hela cells has shown a similar trend. In this case, mitochondria expressing Killer Red™ were exposed to light (559 nm wavelength) to produce mitochondrial ROS. These damaged mitochondria recruited Parkin, and a time course indicated that these mitochondria became devoid of Mfn2 before a decrease in the MOM protein TOM20 or LC3 co-localization (Yang and Yang, 2013).

Another notable observation from Yang and Yang (2013) was that there were foci of ubiquitination on damaged mitochondria that overlapped with ER contact sites. Mfn2 is

known to be located at mitochondria-ER contact sites (Hoppins *et al*., 2011; Zorzano *et al*., 2010). Therefore, the ubiquitination of Mfn2 and subsequent proteasomal degradation may disconnect damaged mitochondria from the ER, thus promoting efficient removal of such mitochondria and preventing the dangerous combination of damaged mitochondria and calcium.

CONCLUSIONS AND PERSPECTIVES

PINK1/Parkin-mediated mitophagy in proliferating cells results in the rapid ubiquitination of the Mfns and their subsequent degradation by the proteasome. Given the central role mitochondrial dynamics has in the segregation of damaged mitochondria it is perhaps not surprising that the protein levels of these two fusion proteins are closely regulated. It is still unclear whether the Mfns' role in mitophagy is solely confined to regulation of mitochondrial fusion, or whether Mfn2 in particular has additional functions. A recent report has highlighted that PINK1 phosphorylates Mfn2, but not Mfn1, which then acts as a receptor for Parkin localization to the MOM (Chen and Dorn, 2013). The authors also presented data that mitochondrial ubiquitination and recruitment of p62 was impaired in the hearts of Mfn2-deficient mice. There is contradictory evidence for the Mfns playing a role in Parkin recruitment. Mfn1 and Mfn2 null MEFs have been reported to recruit Parkin to depolarized mitochondria in a similar fashion to normal MEFS (Narendra *et al*., 2008), while Parkin localization was partially impaired in insect cells following *dMfn* RNAi (Ziviani *et al*., 2010).

The vast majority of published reports on PINK-1/Parkin-mediated mitophagy have used CCCP and proliferating cells. The development of other protocols to induce mitophagy is vital to show that the ubiquitination of the Mfns and other MOM is not restricted to this model (Yang and Yang, 2013). It is also important to remember that mitochondrial physiology and dynamics varies widely between cell and tissue types, and therefore it is likely that there will be differences in mitophagy as well. For example, mitochondrial networks in cardiomyocytes undergo fission much more slowly than cultured cancer cell lines (Dorn, 2013). Cancer cell lines such as Hela can derive their energy needs from glycolysis, while neurons rely heavily on mitochondrial respiration and will be exposed to different local environments in synapses, axons, and the soma (Van Laar *et al*., 2011). Furthermore, synaptic mitochondria have to be transported over relatively large distances for degradation in the soma. While there is evidence that PINK1/Parkin dependent mitophagy and Mfn ubiquitination does occur in neurons, the kinetics would appear to be much slower than in proliferating cells. Intriguingly, Van Laar *et al*. (2011) have shown that when Hela cells were forced to rely on mitochondrial respiration like neurons (by culturing them in galactose-containing media rather than glucose), Parkin localization to depolarized mitochondria was not detected after 3 hours.

Impairment of PINK1/Parkin-mediated mitophagy has provided a potential explanation for the mitochondrial dysfunction observed in cellular and animal models of PINK1 or Parkin deficiency (Gegg *et al*., 2010; Park *et al*., 2006; Schapira and Gegg, 2011; Ziviani *et al*., 2010) and cardiomyocytes lacking Mfn2 (Chen and Dorn, 2013). The observation that the decreased oxidative phosphorylation in PINK1 deficient cells could be reversed by overexpressing Parkin, and thus improving mitophagy (Gegg *et al*., 2010), has highlighted that

therapeutic strategies to improve mitophagy may be successful in treating these familial forms of PD. Furthermore, since there is an accumulation of mutated mtDNA and evidence of decreased mitochondrial and lysosomal function in both aging and sporadic PD (Bender *et al*., 2006; Schapira and Gegg, 2011; Terman *et al*., 2010), strategies to improve mitophagy or the autophagy-lysosomal pathway could have more widespread beneficial effects.

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CHAPTER

13

Mitochondrial Alterations and Mitophagy in Response to 6-Hydroxydopamine

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OUTLINE

Abstract

6-Hydroxydopamine (6-OHDA) is a hydroxylated analogue of dopamine that has been exploited as an experimental model to study Parkinson's disease (PD). In this study, we highlight the participation and relevance of mitochondrial alterations and mitophagy in response to 6-OHDA. Mitochondria play important roles in life and death of eukaryotic cell processes via oxidative phosphorylation. Mitochondrial dysfunction, either functional or morphological, has been widely associated with the cytotoxic effect of 6-OHDA. PD patients present deficiencies in the oxidative phosphorylation system. Complex I activities were found to be significantly reduced in postmortem substantia nigra of PD patients. In line with this, 6-OHDA diminishes complex I and IV activities. Mitochondrial membrane permeabilization, a critical event during apoptosis that leads to the release of cytochrome c from mitochondria, is induced by 6-OHDA. We also discuss the effect of 6-OHDA on morphological mitochondrial dynamics processes. Irreversible mitochondrial swelling does not take place in this model. The pro-apoptotic protein Bax is responsible for mitochondrial cytochrome c release. 6-OHDA induced mitochondrial fragmentation by a mechanism in which the mitochondrial fission machinery is involved. Furthermore, Drp1 translocates from the cytosol to the mitochondria in the presence of 6-OHDA. Finally, we discuss the role of reactive oxygen species as second messengers in these processes.

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INTRODUCTION

Parkinson's disease (PD) is a progressive and neurodegenerative condition. It is characterized primarily by the loss of dopaminergic neurons in the substantia nigra pars compacta leading to a dopamine deficit in the striatum. Nowadays, PD is the second most common neurodegenerative disease, just after Alzheimer's disease. The main symptoms in PD are bradykinesia, rigidity, slow motion, dopaminergic neuron death, and the presence of Lewy bodies in the surviving neurons. Little is known about the etiopathogenesis of PD. The sporadic form of PD seems to be a complex multifactorial disorder with variable contributions of environmental factors and genetic predisposure. In the same way as for many other diseases, we have preclinical models to understand the underlying molecular and cellular mechanisms that cause PD. Such models will also help to identify novel potential drug targets, and to elucidate the effects of new drug candidates. 6-Hydroxydopamine (6-OHDA), also known as oxidopamine, or 2,4,5-trihydroxyphenethylamine ($C_8H_{11}NO_3$), is a toxic oxidative metabolite of dopamine that has been exploited broadly to generate experimental models of PD. It also has been used extensively as a test system for novel symptomatic agents, and for assessment of neuroprotective and neurorepair strategies. 6-OHDA-lesioned rats appear to be a good model for predicting the efficacy of new dopaminergic drugs that enter phase II/III clinical trials.

MITOCHONDRIAL ALTERATIONS

Mitochondria play critical roles in regulating cellular viability and show characteristic vulnerability to injury. They can be considered as headquarters where the cell controls signaling pathways that under some circumstances can lead to cell death (Jordan *et al.*, 2003, 2011). Mitochondrial alterations have been reported in PD. We are going to classify them in two main groups: biochemical and morphological alterations.

Biochemical Mitochondrial Alterations

The most important mitochondrial energy-yielding reaction is performed by the oxidative phosphorylation system (OXPHOS). This system can be resolved into five large multisubunit complexes (CI–CV), and is embedded in the inner mitochondrial membrane. Electron transfer from OXPHOS substrates to molecular oxygen results in the translocation of protons across the inner mitochondrial membrane at CI, CIII, and CIV, creating a substantial electrochemical gradient. This gradient is utilized for ATP synthesis, ion translocation, and protein import. Mitochondrial dysfunction in PD has been demonstrated as a reduction in complex I activity in the brains of patients and laboratory animals. Complex I, also known as NADH–ubiquinone oxidoreductase, is a multi-subunit integral membrane complex of the mitochondrial electron transport chain that catalyzes electron transfer from NADH to ubiquinone. The redox reaction is coupled to proton translocation across the membrane, contributing to the proton motive force. The activity of this enzyme complex is considered the rate-limiting step for the mitochondrial respiratory chain, and therefore is an important factor in the regulation of oxidative phosphorylation. Complex I is also considered an

important site of superoxide generation in mitochondria and, thus, a potential source of reactive oxygen species (ROS) during aging processes. Complex I activities were reported to be significantly reduced (in the range of 30%) in postmortem substantia nigra and platelets of PD patients. On the other hand, complex I activity data from skeletal muscle and other nonneuronal tissues were not consistent. Although the complex I deficiency was first reported to be specific for the substantia nigra, recently a 30% reduction in complex I activity was also detected in frontal cortex mitochondria from PD postmortem brain. Interestingly, Keeney *et al*. (2006) demonstrated that catalytic subunits of complex I derived from PD frontal cortex mitochondria are oxidatively damaged, correlating with complex I misassembly and dysfunction. Whether this increase in oxidative damage is specific for complex I, and which subunits are affected, have not been addressed so far. In conclusion, several lines of evidence substantiated a link between PD and complex I activity. Conflicting reports on complex I activities in PD patients may best be explained by methodological issues, in particular, sample preparation and assay techniques. It is also conceivable that variations in the extent of complex I deficiency, or differences between brain and peripheral tissues, reflect the fact that PD is not a uniform entity, which, rather than that, is an etiologically and pathologically heterogeneous syndrome. Recently, it has been shown that 6-OHDA affects mitochondrial respiration by causing a reduction in both the respiratory control ratio and state 3 respiration, with no significant effects on state 4 (Iglesias-Gonzalez *et al*., 2012).

6-OHDA may act as a direct inhibitor of the mitochondrial respiratory chain complexes. The most important complex that gets destroyed in the substantia nigra is CoQ10 (coenzyme Q10 complex) which is found in the mitochondria along with cardiolipin. The destruction of CoQ10 would be reduced if an ample amount of cardiolipin were available. Since cardiolipin forms a component of Complex 3 and Complex 4, it will be oxidized first rather than CoQ10. Results from different laboratories have shown that cardiolipin molecules are specifically bound to complex I of the respiratory chain and required for its functional activity. Cardiolipin, also known as diphosphatidylglycerol, is an anionic phospholipid. It is unique in that it contains four, mostly highly unsaturated, acyl chains and two negative charges on the head group. Cardiolipin is almost exclusively found in mitochondrial membranes, where it is predominantly located in the inner mitochondrial membrane although it is also found at the contact sites between the inner and outer membranes (Ardail *et al*., 1990).

Cardiolipin has been implicated in the process of apoptosis in animal cells through its interactions with a variety of death-inducing proteins, including cytochrome c. The enzyme is believed to act as a peroxidase, which reacts quite specifically with cardiolipin causing oxidation and then hydrolysis of the product cardiolipin hydroperoxides. The consequence is that cytochrome c is released into the intermembrane space, while the oxidized cardiolipin is translocated to the outer mitochondrial membrane and participates in the formation of the mitochondrial permeability transition pore that facilitates egress of proapoptopic factors from mitochondria into the cytosol, where they trigger apoptosis.

Morphological Mitochondrial Alterations

Aside from their important role as an energy source, mitochondria are also critically involved in many cellular processes. Mitochondrial membrane permeabilization is a critical event during apoptosis and represents the point-of-no-return of this lethal process

(Galluzzi *et al.*, 2009). Permeabilization of the mitochondrial outer membrane (MOMP) is a crucial step in both apoptosis and necrosis. This phenomenon allows the release of mitochondrial death factors, which facilitates or triggers different signaling cascades. Ultimately, this causes the execution of the cell, involving release of cytochrome c. Treatment with 6-OHDA induced the release of cytochrome c from brain mitochondria (Gomez-Lazaro *et al.*, 2008b). MOMP is regulated either by the formation of the permeability transition pore (PTP) or by the insertion of Bcl-2 family members into the MOM. There is some controversy about the role of the PTP in the 6-OHDA model. The PTP is a nonspecific large proteinaceous pore, spanning both mitochondrial membranes. It allows the passage of ions and substrates at the cost of loss of mitochondrial membrane potential. This leads to ATP depletion and energetic collapse, and thus contributes to cell death (Qian *et al.*, 1999). Onset of a PTP is such a severe perturbation of mitochondrial function that it basically assures cell death. We have demonstrated that 6-OHDA did not induce PTP formation (Gomez-Lazaro *et al.*, 2008b). First, 6-OHDA did not decrease the levels of mitochondrial calcein fluorescence, a hallmark of PTP opening. Second, 6-OHDA-induced mitochondrial cytochrome c release was not inhibited in cell cultures that were pretreated with the PTP inhibitor cyclosporin A. Third, a mitochondria suspension isolated from brain did not readily undergo swelling upon exposure to 6-OHDA.

6-OHDA AND MITOCHONDRIAL DYNAMICS

Recent research is building a consensus that mitochondrial structure and dynamics are actively and tightly controlled by cellular stimuli, signaling events, and perturbations inside this organelle (for a review, see Perkins *et al.*, 2009). In neurodegenerative diseases such as PD, this equilibrium is clearly broken and an increment in fission is observed (Gomez-Lazaro *et al.*, 2008a; Solesio *et al.*, 2012). More detailed, upon 6-OHDA treatment, SH-SY5Y cell cultures showed a significant increase in the percentage of cells with truncated or fragmented mitochondria. This process was dependent on the mitochondrial fission factor dynamin-1-like protein (Drp1 or Dlp1). Drp1, a cytosolic dynamin protein with GPTase activity, migrates to the outer mitochondrial membrane upon a fission trigger (Smirnova *et al.*, 2001) and, once there, Drp1 constricts the organelle. We observed that 6-OHDA recruited Drp1 to mitochondria. Confocal microscopy analysis of cell cultures overexpressing Drp1-GFP revealed that addition of 6-OHDA modified the cytosolic and diffuse distribution of Drp1, leading to a punctuate mitochondrial distribution. The relevance of this protein for mitochondrial fission was evidenced further by pretreating cells with the mitochondrial division inhibitor-1 (mdivi-1). mdivi-1 prevents docking of Drp1 at the mitochondrial outer membrane and, consequently, reduces mitochondrial fission after several insults. Consistent with this, we observed that mdivi-1 significantly attenuated 6-OHDA-induced mitochondrial fission. Upon addition of 6-OHDA, the pro-apoptotic protein Bax also translocated from the cytosol to the mitochondria in SH-SY5Y cells. In our experimental model, this process took place after mitochondrial fragmentation and Drp1 translocation. SH-SY5Y cells consistently showed mitochondrial Bax localization 6 h after 6-OHDA addition. In agreement with this, in $Drp1^{-/-}$ cells or in cells that were transfected with a dominant negative allele (DrpK38A) that is defective in GTP binding, Bax translocates to the mitochondria

with kinetics similar to that observed in wild-type cells. Moreover, mitochondrial release of pro-apoptotic proteins, such as Smac/DIABLO, is not affected by the absence of Drp1. Accordingly, Bax is dispensable for mitochondrial fission in the 6-OHDA model, but also when fission is induced by hFis1 or by a widely used intrinsic apoptotic stimulus (Gomez-Lazaro *et al.*, 2008a).

6-OHDA AND AUTOPHAGY

Autophagy was described for the first time in 1963 by de Duve and collaborators (de Duve, 1963). Autophagy, or cellular self-digestion, is a catabolic process, which directs cell constituents (damaged organelles, unfolded proteins, intracellular pathogens) to lysosomes for degradation. Under basal conditions, autophagy is involved in the degradation of long-lived proteins, whereas another catabolic process, the ubiquitin–proteasome pathway, is responsible for the degradation of short-lived proteins. Three types of autophagy have been identified: macroautophagy, chaperone-mediated autophagy, and microautophagy. Macroautophagy (hereafter referred to as autophagy) involves the formation of a small vesicular sac called the isolation membrane or phagophore. The hallmark of autophagy is the emergence of double-membrane vesicles, which are called autophagosomes. The autophagosomes engulf a portion of the cytosol and, subsequently, translocate towards and fuse with lysosomes. It has been shown that induction of autophagy can occur by many environmental stimuli, for instance oxidative stress and nutrient depletion. But the relevance of autophagy in cell fate remains unknown and controversial. Under some circumstances, activation of autophagy has been reported to be potentially cytoprotective, including during neurodegenerative disorders. Basal levels of autophagy are essential for neuronal survival, and accordingly autophagic dysfunction has been linked to a growing number of neurodegenerative disorders (Levine and Kroemer, 2008). Previous results from our laboratory showed that autophagy is induced by 6-OHDA treatment (Solesio *et al.*, 2012). The 6-OHDA-induced autophagy correlated with an increase in the microtubule-associated protein-1 light chain 3 (LC3-II) level, and with the accumulation of autophagic vacuoles in the cytoplasm. Consistent with these data, accumulation of autophagic vacuoles and activation of lysosomes has been observed in nigral rat neurons, which had been treated with 6-OHDA (Li *et al.*, 2011). In 6-OHDA-challenged SH-SY5Y cells, the formation of autophagosomes took place distant from the mitochondria, and in a time-dependent fashion. The autophagosomes specifically approached fragmented mitochondria, as indicated by the observed co-localization at the later time points of 6-OHDA treatment. In line with this, the mitochondrial quality control hypothesis postulates that dysfunctional mitochondria are susceptible to degradation (Twig *et al.*, 2008).

The term "mitophagy" has been used to describe two different types of mitochondrial degradation in mammalian systems: (i) the selective removal of damaged or dysfunctional mitochondria for cellular maintenance, and (ii) the complete or partial clearance of mitochondria by autophagy during cell differentiation. Mitophagy plays crucial roles in maintaining mitochondrial homeostasis and in the complete/partial elimination of mitochondria. Indeed, it is the process that allows cells to eliminate damaged mitochondria, and can play a prosurvival or a prodeath role. Depolarized mitochondria are not able to go into

the fission–fusion cycle; they are marked as unable mitochondria, and then eliminated by mitophagic events (Twig *et al.*, 2008).

The responsible pathway for the induction of autophagy is not entirely clear (Figure 13.1). Reactive oxygen species are second messengers involved in the toxic pathways activated by 6-OHDA. When the production of ROS exceeds degradation, increased ROS levels may induce oxidative stress, which causes damage to cellular components, including DNA, proteins, and lipids. A rise in ROS levels might activate autophagy as a protective mechanism. Indeed, upregulation of autophagy tends to reduce the ROS levels, and prevents the deleterious effects of elevated ROS levels. The cellular components that are damaged by oxidative stress are sequestered by autophagosomes. Thus, under such stress conditions, regulatory proteins must be activated to stimulate the autophagic machinery. Consistent with this hypothesis, we have demonstrated a relationship between ROS production (H_2O_2 increase in cells) and 6-OHDA-induced mitochondrial fission, and subsequently, mitophagy (Solesio *et al.*, 2012, 2013). Interestingly, this 6-OHDA-induced H_2O_2 increase in the cells seems to occur upstream of mitochondrial fission, as inhibition of this dynamic process by mdivi-1 did not block mitochondrial H_2O_2 production. Consequently, autophagy is initiated by ROS in a manner that is sensitive to antioxidants. Therefore, most avenues for therapeutic targeting of mitochondria so far have focused on counteracting ROS production. For instance, TEMPOL and MnTBAP, two well-known antioxidant drugs, abolished translocation of Drp1 to mitochondria and, consequently, 6-OHDA-induced mitochondrial fission. In keeping with this interpretation, oxidative stress might be responsible for induced mitochondrial fission in several processes, including PD, perhaps due to a posttranslational redox change in the Drp1 protein (Nakamura and Lipton, 2010, 2011). Different data suggest an involvement of p38 mitogen-activated protein kinase, mammalian target of rapamycin (mTOR), serine/threonine kinase Akt, and 5-AMP-activated protein kinase alpha in autophagy. The induction of autophagy by mTOR inhibition under starvation conditions is a well-known phenomenon in mammalian cells. mTOR is the mammalian ortholog of the yeast protein kinase TOR that negatively regulates autophagy. Various pathways and small molecules regulating autophagy via mTOR and mTOR-independent mechanisms have been identified in recent years. The activity of mTORC1 can be inhibited by rapamycin (sirolimus), a lipophilic macrolide antibiotic first isolated from *Streptomyces hygroscopicus*. Rapamycin is a potent autophagy inducer in various cell lines from yeast to mammalian cells, including neurons. In mammalian cells, rapamycin forms a complex with the immunophilin FK506-binding protein of 12 kDa (FKBP12), which then stabilizes the raptor-mTOR association and inhibits the kinase activity of mTOR. Interestingly, these mechanisms frequently are modulated by 6-OHDA. 6-OHDA induced phosphorylation of AMPK and its target Raptor, followed by the dephosphorylation of mTOR and its substrate p70S6 kinase (S6K). Supporting this, rapamycin protects against neuron death in *in vitro* and *in vivo* models of PD (Malagelada *et al.*, 2010). Several classes of small molecular antioxidant mimetics have been shown to protect against central nervous system injuries, such as dopaminergic neuron degeneration. MitoQ is nontoxic and orally bioavailable in animal models and humans. Recently, we have shown how MitoQ blocks 6-OHDA-induced mitochondrial fission. Our data support the notion that MitoQ blocks the activation of the mitochondrial fission machinery by preventing mitochondrial translocation of Drp1 (Solesio *et al.*, 2013). On the other hand, we have shown how pyruvate protects cerebellar granular cells from 6-hydroxydopamine-induced

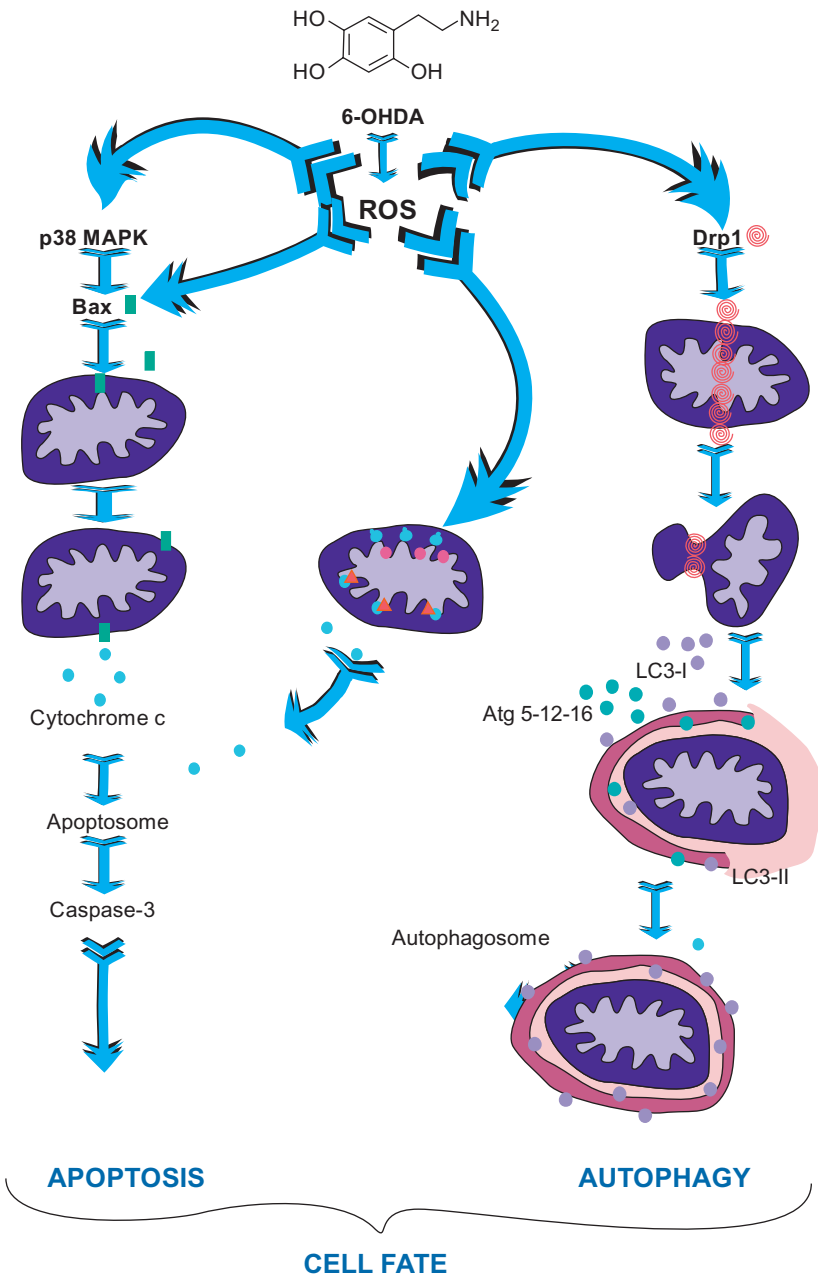

FIGURE 13.1 A hypothetical model of mitochondrial alterations and mitophagy in response to 6-hydroxydopamine. Oxidative stress triggered by 6-OHDA causes subsequent Drp1 and Bax mitochondrial translocation. Abbreviations: 6-OHDA, 6-hydroxydopamine; Drp1, dynamin-1-like protein; LC3-II, microtubule-associated protein-1 light chain 3; ROS, reactive oxygen species.

cytotoxicity by activating the Akt signaling pathway (Fernandez-Gomez *et al.*, 2006) or how in SH-SY5Y cells challenged with 6-OHDA an increase in the levels of phosphorylated form of p38 MAPK take place. It remains to be determined whether the induction of autophagy by 6-OHDA is related to cell death or to a cytoprotective response that is activated by dying cells in order to cope with stress. In a previous study, tyrosine hydrolase-positive neurons in substantia nigra were protected from 6-OHDA-induced cell death when they were pretreated with the autophagy inhibitor 3-methyladenine. On the other hand, experiments using neuron-specific knockout mouse models have demonstrated that autophagy deficiency leads to protein aggregation and neurodegeneration, even in the absence of disease-related aggregate-prone proteins (He and Klionsky, 2006).

In conclusion, this work presents a short overview of mitochondrial dynamics (especially focusing on fission and mitophagy) in the 6-OHDA-induced pharmacological model used in PD. Important questions – for example, how are mitochondrial alterations coordinated and how is this integrated into basic physiological processes such as apoptosis and mitophagy – remain to be answered in detail. It is noteworthy that some of the above-described 6-OHDA-modulated events also take place in other PD models, such as those using different drugs. For instance, Rotenone (Blesa *et al.*, 2012), Paraquat (Kim and Choi, 2008), and MPP^+ (Zhu *et al.*, 2007), which act by inhibiting complex I from OXPHOS, are able to cause mitochondrial fragmentation and autophagy.

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CHAPTER

14

Role of Mitochondrial Fission and Mitophagy in Parkinson's Disease

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OUTLINE

Abstract

The involvement of mitochondrial dysfunction as a causal factor of Parkinson's disease (PD) is well established. Impaired mitochondrial function is a predominant feature of PD. Here, we discuss mitochondrial fission and mitophagy, two processes that are described in PD. In experimental models as well as in samples from PD patients, mitochondrial fission has been shown to occur as an early step during these neurodegenerative processes. In these processes a machinery of proteins actively participates, including dynamin related protein 1 and fish. The deleterious effects of oxidative stress, resulting from increased levels of reactive oxygen species, including membrane and protein damage, are well recognized as important inducers of mitochondrial fission. Here, we discuss the current knowledge and prevailing hypotheses regarding

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mitochondrial dysfunction in either genetic or sporadic PD. While many questions remain unanswered, the prevalence of mitochondrial fission as an early event in neurodegenerative diseases warrants further exploration of all of these areas for development of potential treatments. Understanding the nature of these events, how they are activated, and their relative contributions to PD-mediated toxicity may strengthen future studies that aim to develop therapeutic prevention and intervention.

INTRODUCTION

Parkinson's disease (PD) is the most common neurodegenerative movement disorder and the second most common neurodegenerative disease. PD patients suffer from resting tremor, bradykinesia, muscle rigidity and postural instability. PD is a multifactorial disease, produced by several failures in different biochemical systems. Most of the PD cases are sporadic, although familial PD with autosomal dominant or autosomal recessive mutations also account for PD cases. Among the pathological hallmarks of the disorder are Lewy bodies, cytoplasmic inclusions that contain aggregated proteins, and degeneration of substantia nigra dopamine neurons.

The involvement of mitochondrial dysfunction as a causal factor of PD is well supported by observations in patients (Dauer and Przedborski, 2003; de Rijk *et al.*, 1997). Causal factors include oxidative stress, mitochondrial dysfunction, protein aggregation, and abnormality with in the ubiquitin-proteasome pathway (Levy *et al.*, 2009). Along with ubiquitinated protein aggregates, affected neurons often contain structures related to autophagy. In fact, an increased number of autophagic vacuoles and related structures of autophagy have been found in PD (Anglade *et al.*, 1997) and experimental models of PD (Solesio *et al.*, 2012). Herein we review the relationship between PD and mitochondrial dynamics, especially with mitochondrial fission and mitophagy. Particular focus is applied to the participation of parkinsonian gene mutations and sporadic PD.

MITOCHONDRIAL FISSION AND PD

In the past, mitochondria were regarded as static and rigid systems; this view was influenced by the black-and-white pictures obtained by old electron microscopy. But nowadays we are familiar with mitochondria as being highly dynamic systems, undergoing continuous fission and fusion cycles. All of these processes are referred to as mitochondrial dynamics, which results in a delicate equilibrium in healthy cells (Westermann, 2010).

Imbalances in mitochondrial dynamics have been widely reported in pathogenesis, being especially important in the case of neurons, where we find high energy demand. In neurons, the mitochondrial fission/fusion machinery is intimately and critically involved in the formation of synapses and dendritic spines. Neurons are more vulnerable to degeneration and require high energy demands and so they are particularly dependent on suitable mitochondrial dynamics. Consequently, disrupted mitochondrial dynamic processes might participate in neurodegenerative diseases including PD. The fission of preexisting organelles is essential for generation of the new mitochondria; any disruption of their balance could change the steady-state distribution of mitochondrial span.

A large GTPase in the dynamin family, DRP1/Dnm1 (human/yeast nomenclature), is required for mitochondrial fission. DRP1/Dnm1 assembles from the cytosol onto mitochondria at focal sites of division, forming spiral chains around membrane constriction sites. Dnm1 assembly facilitates GTP hydrolysis and thereby organelle fission. During apoptosis, DRP1 foci accumulate on mitochondria and mediate dramatic mitochondrial fission prior to caspase activation. Because Drp1 lacks a mitochondrial targeting sequence, some protein(s) may be needed to recruit Drp1 to mitochondria. Human Fis1 is a good candidate for this recruitment because in yeast, Fis1p is required for the proper assembly and distribution of Dnm1p-containing complexes on mitochondria. The structure of hFis1 resembles that of certain proteins involved in mitochondrial import. In this line of Fis functions in the mitochondrial fission machinery are data from our laboratory, where overexpression of h-Fish in the dopaminergic neuroblastoma SH-SY5Y cells results in mitochondrial morphology alterations. Cassidy-Stone *et al.*, 2008 have identified an inhibitor of mitochondrial division, called mitochondrial division inhibitor-1 (mdivi-1), using yeast screens of chemical libraries. mdivi-1 inhibits Drp1 assembly and GTPase activity *in vitro*. Interestingly, recent work in our laboratory has shown that the SY-SY5Y cells treated with mdivi-1 present a lower number of fragmented mitochondria when challenged with 6-hydroxydopamine (6-OHDA) (Solesio *et al.*, 2012).

Cells have developed sophisticated systems to deal with the diverse challenges imposed on mitochondrial functional integrity. These systems could comprise a "multistep" mitochondrial quality control network that assists in the spatial segregation of damaged mitochondria. The first tier of quality control is provided by both molecular chaperones and the intracellular proteolytic system, which selectively remove excess and damaged proteins from the mitochondrial outer membrane. A second step in mitochondrial quality control could be mediated by fusion of damaged mitochondria with neighboring mitochondria. However, severe injury of mitochondria impairs fusion and further activates fission-dependent fragmentation and sequestration by an autophagic process, termed mitophagy. Accumulating data suggests that mitochondrial dysfunction by itself triggers mitophagy (Kim *et al.*, 2007).

MITOPHAGY

The autophagic pathway is responsible for the degradation of long-lived proteins, protein aggregates and cytoplasmic organelles. Degradation of proteins and organelles begins with the sequestration of cargo into various vesicles of the autophagic pathway. Autophagic degradation leads to the recycling of biological macromolecules, which are released for use in biosynthetic pathways (Yang *et al.*, 2006). Given the role of the autophagic pathway as a degradation system for clearance of aggregated proteins, it is not surprising that autophagic deregulation observed in various neurodegenerative disorders has attracted mounting interest.

Autophagy can be classified into three groups: macroautophagy, microautophagy, and autophagy mediated by chaperones. The first one is the most common and when we find the term "autophagy" in scientific literature it is often referred to as "macroautophagy." In this process, after autophagosome formation and maturation there is a fusion between it and lysosomes, in order to degrade its internal content. This process is regulated by more

than 30 kinds of autophagy-related gene proteins, also called Atg, which are highly conserved from yeast to humans.

While autophagy is required for homeostasis in all cell types, nondividing cells such as neurons are particularly sensitive to changes in autophagic degradation. As most neurons must survive for the lifetime of the organism, maintenance of organelle function and clearance of aberrant or damaged proteins are critical processes regulated by autophagy. As a primary protective mechanism that maintains nutrient and energy homeostasis in response to stress, deregulation of autophagy underlies the pathophysiologies of many diseases. Increasing evidence suggests that deregulation of autophagy results in the accumulation of abnormal proteins and/or damaged organelles, which is commonly observed in neurodegenerative diseases, such as Alzheimer's, Huntington's, and Parkinson's diseases (Banerjee *et al.*, 2010).

The role of autophagy as an alternative cell death mechanism has, in recent years, been a topic of debate. It was the manifestation of autophagosomes in dying cells exhibiting features distinct from apoptosis that led scientists to suggest a death-promoting role for autophagy. However, caution should be exercised when describing the involvement of autophagy in cell death since there is still no concrete evidence showing that autophagy directly executes cell death. Specifically, the use of the phrase "autophagic cell death" is now deemed inaccurate and the Nomenclature Committee of Cell Death 2009 has advised the use of the term "cell death with autophagy" as a more appropriate reference to cell death connected with autophagy (Galluzzi *et al.*, 2009).

In fact, mitochondria were first detected inside an autophagosome in the 1950s. Studies suggest that the selective removal of mitochondria, especially damaged mitochondria, is part of an important homeostatic pathway for organelle quality control. Today we know that mitophagy is regulated by genes implicated in macroautophagy, as well as by its own mitochondrial degradation system, which is less known. Mitophagy is activated through a constitutive and inducible system and it represents a critical point in mitochondrial quantity and quality control. This strict control is very important in the case of mitochondria because these organelles do not have compact chromatin or histones. Pioneering studies in yeast have demonstrated that autophagic degradation of mitochondria, mitophagy, can be a highly selective and tightly regulated process. In yeast cells, mitophagy fits with the common model of a receptor-adaptor system for the selective degradation of a specific cargo by autophagy; a tag on the cargo is recognized by a receptor and/or adapter, which links the cargo with the autophagy machinery via interaction with Atg8. In the case of mitophagy, yeast genetic screens discovered a mitochondrial outer membrane resident protein, Atg32, which functions as the receptor for the sequestration of mitochondria into an autophagosome. During mitophagy, Atg32 is recognized by an adaptor protein, Atg11, which is proposed to play a role in mediating cargo recognition and transport to the phagophore assembly site, the nucleating structure for generation of the phagophore. Mitochondrial fragments containing Atg32 are then enwrapped by the expanding phagophore, ultimately being incorporated into an autophagosome.

Autophagy can function in cell death by execution of a self-killing program of irreversibly injured cells. In some extreme instances of programmed cell death, cells can be completely degraded through autophagic digestion. Accumulating evidence demonstrates that late-stage neuronal cell loss generally occurs via autophagy and it has been suggested that

overactivation of autophagy in neurons is the eventual cause of "physiological" death. An increased number of autophagic vacuoles is responsible for the neuronal cell death, but an alternative view is emerging that autophagy is induced to protect neurons by enhancing degradation of abnormal proteins that might trigger injury or apoptosis in the early stages of cell death (Bandyopadhyay and Cuervo, 2007; Butler and Bahr, 2006). Furthermore, ultrastructural examination indeed revealed "autophagic stress" in melanized neurons of the SNpc in PD patients (Anglade *et al.*, 1997). Moreover, accumulated autophagosomes have been observed in human PD nigral neurons, but not in nigral neurons during normal aging (Anglade *et al.*, 1997).

Mitochondria-derived ROS, at low concentrations, may act as signaling molecules and trigger mitophagy throughout redox regulation of Atg4, an essential cysteine protease in the autophagic pathway. Similarly, Gomes and Scorrano also provided evidence that the profission mitochondrial protein Fis1 induces mitochondrial fragmentation and enhances mitophagy. Nevertheless, these changes were correlated with mitochondrial dysfunction rather than with fragmentation (Gomes and Scorrano, 2008). In addition, mitochondrial fission was also shown to be an important step for the autophagic clearance of depolarized or damaged mitochondria, since overexpression of Drp1 promotes mitophagy.

GENETIC FACTORS

Increasing knowledge of genetics has allowed the scientific community to find genes whose mutations are responsible for a small group of PD, approximately 10% of total patients. These mutations can be dominant or recessive autosomal. They are concentrated in six genes, with, apparently, different functions, but with additive effects between these gene mutations in the production of mitochondrial dysfunctions. These genes are: *α-synuclein*, *leucine-rich repeat kinase 2* (*LRRK2*), the E3 ubiquitin-ligase *Parkin*, the *DJ-1* putative chaperone (Bonifati *et al.*, 2003), the *PTEN-induced putative kinase 1* (*PINK1*) (Valente *et al.*, 2004), and the *Omi/HtrA2* mitochondrial serine protease. In the first two, mutations are dominant, while in the rest of the genes they are recessive.

α-Synuclein

α-Synuclein is a 140 amino acid ubiquitous protein, widely distributed in the brain. In neurons, α-synuclein reaches about 0.5–1% of total proteins (Iwai *et al.*, 1995) and is associated with synaptic vesicles, playing a role in neurotransmitter liberation modulation. α-Synuclein has a tendency to aggregate. Soluble α-synuclein is degraded by the ubiquitin-proteasome system pathway and by chaperone-mediated autophagy (CMA) (Cuervo *et al.*, 2004). In neurons, α-synuclein toxicity seems to be associated with its aggregation, forming small neurotoxic oligomers that develop long and insoluble deposits, the Lewy bodies, affecting mitochondrial metabolism (Siddiqui *et al.*, 2012) and decreasing macroautophagy efficiency.

α-Synuclein wild-type protein expression may inhibit autophagy and contribute to many different symptoms found in PD patients, including abnormal protein aggregation, mitochondrial abnormalities, increased levels of ROS and enhanced susceptibility to

apoptosis. In fact, there seem to be intricate cross-regulation cycles between α-synuclein and autophagy, which complicates much of the study of this pathway, because of the high amount of feedback implicated.

Studies in the substantia nigra of PD brains show that insoluble α-synuclein and α-synuclein aggregates interact with the proteasome leading to its inhibition, an observation also corroborated by assays performed in yeast and cell line models (Webb *et al.*, 2003). Mutant and dopamine-oxidized forms of α-synuclein also impair the CMA pathway leading to an upregulation of autophagy (Chu, 2011; Lynch-Day *et al.*, 2012). Thus, dysfunctional clearance of α-synuclein and, consequently, impairment of protein quality control systems, particularly autophagy, have become prominent pathogenic mechanisms underlying neurodegeneration (Chu, 2011).

Oxidative stress, including the neurotransmitter dopamine, has been linked to increased α-synuclein aggregation. Dopamine itself may contribute to the toxic effects of α-synuclein *in vitro*. α-Synuclein expression can enhance sensitivity to oxidative and nitrative stressors, although it can also be protective in some situations.

Mutations of α-synuclein and the increase of intracellular concentrations of nonmutant α-synuclein have been implicated in the pathogenesis of PD. A classical α-synuclein gene mutation consists of changing alanine for proline in three possible different positions. Besides these classical mutations, families with α-synuclein gene duplicate or triplicate show earlier disease onset and more severity in symptoms.

An association between α-synuclein and mitochondrial morphology has been described, yielding mitochondrial fission by inhibition of the fusion process. α-Synuclein overexpression results in abnormally large mitochondria displaying vacuolization of the cristae. α-Synuclein has an ability to associate with proteins from complex I and the adenylate translocator, impairing their activity. α-Synuclein transgenic mice developed mitochondrial pathology, as mitochondria containing α-synuclein were shrunken, swollen, or vacuolated (Martin *et al.*, 2006; Zhu *et al.*, 2011).

Mutant A53T α-synuclein-induced neuronal death was also shown to be related to increased mitochondrial autophagy. α-Synuclein exhibits consensus motifs for lysosomal degradation, regulated by different systems from macroautophagy. Autophagy is involved in the elimination of the wild-type form of α-synuclein in neurons. In fact, mutants for α-synuclein are able to bind the receptor for CMA, but are unable to internalize it, inhibiting this degradative pathway (Cuervo *et al.*, 2004). The blockage of CMA activity with mutant forms of α-synuclein not only results in the direct buildup of toxicity in the neuron through the formation of aggregates, but it also prevents the protective activity of the protein myocyte enhancer factor 2D (MEF2D). MEF2D, a transcription factor, is an important player in neuronal survival. Patients with PD show an increase of this protein in brain neurons, and a genetic polymorphism of a related protein (MEF2A) has been linked to Alzheimer's disease. CMA-dependent degradation regulates MEF2D activity. MEF2D is continuously shuttled to the cytosol from the nucleus where it interacts with hsc70. In cells, when CMA is inhibited, an inactive form of the protein accumulates in the cytosol and the amount of protein in the nucleus drops. This inactive form can no longer bind DNA. Wild-type and mutant forms of α-synuclein prevent binding between HSC70 and MEF2D (Yang *et al.*, 2009). This suggests that not only does α-synuclein promote neuronal death through the formation of aggregates, but it also promotes cell death by inhibiting cell survival proteins.

The inhibition produced by α-synuclein overexpression occurs at a very early state of autophagosome formation. This suggests an important regulatory role for this protein in autophagosome synthesis. Moreover, it has been suggested that α-synuclein is able to produce toxicity in PD models through cell secretory pathways inhibition, like mitophagy. This effect seems to be mediated by loss of function in Rab1a, which is a key regulator of the early stages of the secretory pathways.

Leucine-Rich Repeat Kinase 2

Leucine-rich repeat kinase 2 (LRRK2), also known as dardarin, is a gene that encodes a 2.527 amino acid protein, and is located in the membranous structures as a dimer and in the cytosol to a small extent. LRRK2 is involved in the modulation of the neuritic arbor and plays a role in regulating axonal-dendritic polarity of synaptic vesicles. As a large, complex, multidomain protein, LRRK2 comprises kinase and GTPase enzymatic activities and multiple protein interaction and catalytic domains. LRRK2, due to its hypothetical relationship with the scaffolding of motors or motor-associated proteins, could be also implicated in the transport of this organelle through the microtubules, as well as in the synaptic vesicle movement in order to allow proper mitochondrial distribution in neurons and other cell types.

There are more than 50 mutations identified in LRRK2, and at least 16 of them are pathogenic. Most of these mutations affect the kinase activity. Mutations in this gene, which apart from the dominant heritage pathway is able to produce sporadic mutations, are the most common cause of genetic PD, although they have reduced penetrance, especially in the southern countries, such as Spain or Portugal, where we find these mutations represent between 6 and 18% of PD genetic cases and between 3 and 6% of the sporadic ones.

LRRK2 mutations and mitochondrial dysfunction increments in this gene activity as well as PD-linked mutations in LRRK2 cause simplification and shortening of neuritic projections (Cherra *et al.*, 2010), and thus neuronal damage and elimination, through autophagy. After that, there is a clear decrease in mitochondrial length. These data have been obtained from SH-SY5Y cells as well as in primary cortical neurons. Moreover, in HEK-293 cells LRRK2 associated with multivesicular bodies is able to elicit increased autophagosomes, which occurs through the disruption of autophagic flux. And LRRK2 mutations are not only able to induce autophagy but also apoptosis, which can be avoided by overexpressing Parkin.

LRRK2 is able to modulate cell vulnerability to mitochondrial dysfunction, in a *Caenorhabditis elegans* model (Saha *et al.*, 2009). LRRK2 expression is able to produce mitochondrial fragmentation, with Drp1 implication. In fact, PD-associated mutants induce an important increment in Drp1 mitochondrial migration, suggesting an important role for mitochondrial fission. Moreover, a physical interaction between LRRK2 and Drp1 exists, which is enhanced by PD-associated mutations.

LRRK2 interacts with various proteins implicated in the regulation of autophagy, such as CAMKK-β/AMPK, which are dependent on Ca^{2+} and can induce the accumulation of autophagosomes (Gomez-Suaga *et al.*, 2012). In *in vivo* studies, a depletion of LRRK2 is related to a decrease in 4EBP, which is the target of mTOR. This finding directly associates the LRRK2 protein with aging and autophagy processes. However, interestingly, there has

been observed an age-dependent biphasic alteration in autophagic activity in LRRK2 knockout accompanied by modulations in levels of lysosomal proteins and proteases at different months of age.

DJ-1

DJ-1 (CAP1/RS/PARK7) is a 189-amino acid located, at least partially, in mitochondria. DJ-1 is implicated in broad biological functions including antioxidant activity, modulation of transcription, and chaperone-like functions (Cookson, 2003). Mutations in DJ-1 are the basis of 2% of the cases of EP with early input and they are due to protein loss of function. DJ-1 is abundantly expressed in reactive astrocytes, but not in neurons. When DJ-1 is knocking down, there is impairment in mitochondrial lysosomal function, which can be related with the observed decrease in the basal autophagy rate. DJ-1 also appears to play a role in autophagy, a process thought to maintain sufficient ATP during periods of energy limitation and to ensure the functionality of vital cellular processes such as transcription and protein synthesis under conditions of limited oxygen availability. Pisani *et al.* (2006) demonstrated that DJ-1-deficient dopaminergic neurons show enhanced sensitivity to energy metabolism impairment when they assessed the responses of dopaminergic cells to combined oxygen and glucose deprivation.

Parkin

Parkin, the gene product of *PARK2*, is a primarily cytosolic ubiquitin E3 ligase that contains an ubiquitin-like domain. Parkin functions in the cytosol, in the ER, on mitochondrial targets, and at the plasma membrane. More than one hundred mutations in that gene, which include dose alterations and small changes in sequence, have been identified. The *PARK2* gene has been reported to be mutated in nearly 50% of autosomal recessive and 10–15% of sporadic early-onset PD. Parkin protein is involved in the maintenance of mitochondrial morphology (Greene *et al.*, 2003; Palacino *et al.*, 2004). Different phenotypes exist for Parkin deficiencies in different model systems. Apart from its function in mitochondrial morphology, it seems that Parkin plays other roles in mitochondria. As an example, there is a group that proposes an association between this protein and the mitochondrial outer membrane in order to prevent mitochondrial swelling and cell death.

Recent studies from Richard Youle's group and others, however, have provided a model for Parkin's role in eliminating impaired mitochondria (Narendra *et al.*, 2008). Parkin is specifically recruited to damaged mitochondria and promotes their autophagic degradation. Parkin is selectively recruited to mitochondrial fragments that have decreased or no membrane potential, suggesting a role for Parkin in distinguishing between healthy and damaged mitochondria. Parkin-marked mitochondrial fragments are LC3 (a mammalian homologue of yeast Atg8) positive, further demonstrating that clearance of damaged mitochondria occurs through autophagy. Overexpressed Parkin is also recruited to mitochondria upon an increase in complex 1-dependent ROS, which follows treatment with the herbicide paraquat, a toxin frequently used to induce a PD phenotype in some animal and cell culture models.

PTEN-Induced Kinase 1

PTEN-inducted kinase 1 (PINK1) is a cytosolic and mitochondrially localized 581-amino acid serine/threonine kinase that possesses an N-terminal mitochondrial targeting sequence. Mitochondrial localization implicates PINK1 as a potential regulator of mitochondrial processes associated with apoptosis, such as the electron transport chain and opening of mitochondrial permeability transition pores (PTPs). Expression of PINK1 on individual mitochondria is regulated by voltage-dependent proteolysis; thus, low levels of PINK1 are maintained on healthy, polarized mitochondria. In steady-state cells, PINK1 is imported into the mitochondrial inner membrane in a membrane potential-dependent manner. When imported into the inner membrane, the mitochondrial inner membrane rhomboid protease presenilin-associated rhomboid like protein (PARL) mediates the cleavage of PINK1 (Jin *et al.*, 2010). Upon mitochondria depolarization, PINK1 import into the inner membrane is impaired, leading to a rapid PINK1 accumulation on the outer membrane of damaged mitochondria. When it is mutated by big deletions, punctual mutations, or sporadic changes, PD has a very early onset and a slow progression.

Localized in the mitochondrial intermembrane space, PINK1 plays an important role in mitochondrial dynamics machinery regulation (Yang *et al.*, 2008). PINK1 could act as a neuroprotective factor, by phosphorylating other proteins located in mitochondria, as an answer to oxidative stress, avoiding mitochondrial dysfunction. In fact, PINK1 can alter mitochondrial fission/fusion balance and modify mitophagy rate. PINK1 knockdown cells exhibit mitochondrial functional and morphological abnormalities, with enhanced mitophagy. The loss of PINK1 function causes mitochondrial dysfunction and increases cellular susceptibility to stress. PINK1 phosphorylates the downstream effector TRAP1 and the downstream interactor Omi/HtrA2 (PARK13) and plays an essential role in regulation of mitochondrial integrity in the PINK1 related pathway. PINK1 protects against oxidative stress-induced cell death by suppressing cytochrome c release from mitochondria via TRAP1 phosphorylation. In addition, PINK1 regulates the Parkin/IkB kinase (IKK)/NF-κB, PI3-kinase/AKT and HtrA2 signaling pathways.

PINK1 acts upstream of Parkin, both of them playing a key role in the specification of depolarized mitochondria for sequestration in aggresomes and/or autophagosomes. PINK1, as well as Parkin, is necessary for mitochondrial morphology maintenance, playing an essential role in mitophagy activation. Also, we know that the kinase activity of PINK1 is essential to develop mitophagy mediated by PINK1-Parkin. When there is a low membrane potential, an accumulation of PINK1 occurs in the outer mitochondrial membrane, which induces Parkin mitochondrial migration and activation. Then, Parkin is able to ubiquitinate Mfns located in this membrane, which are eliminated by the proteosome. Remaining mitochondria are not able to develop fusion cycles and are eliminated by mitophagy (Kawajiri *et al.*, 2011).

PINK1-deficient neurons show mitochondrial fragmentation and swelling and cristae abnormalities. All these markers increase over time, and some of them could be a consequence of a decrease in mitophagy rate, or they could be related to a decrease in PINK1-deficient mitochondria to buffer calcium. In fibroblasts from patients who are PINK1-defitient, there is a decrease in calcium buffering mitochondrial ability, which could be a consequence of a decrease in mitochondrial refill trough mitophagy, (Dagda *et al.*, 2009).

In fact, PINK1 participates in damaged mitochondrial elimination (Dagda *et al.*, 2009; Geisler *et al.*, 2010; Narendra *et al.*, 2009).

Parkin translocates to mitochondria when these are depolarized, acting as a marker for mitophagy. Moreover, mitochondrial membrane potential decreases by CCCP inducing endogen PINK1 accumulation in mitochondria. If PINK1 is silenced, Parkin is not able to translocate to mitochondria, blocking mitophagy. Thus, PINK1 accumulation in depolarized mitochondria produces Parkin migration and mitophagy.

Moreover, we know that this kind of mitophagy induced by PINK1/Parkin could be mediated by VDAC and the protein p62 has been postulated as a key factor, implicated in mitophagosome elimination. Apart from these proteins, a new study showed that another protein called Nix seems to be essential for mitophagy mediated by PINK1/Parkin.

Omi/HtrA2

Omi/HtrA2 is a serine protease with an N-terminal mitochondrial targeting sequence. The encoded protein is located in mitochondrial membrane and in the intermembrane space. Mutations in the Omi/HtrA2 gene as causes of PD have been described. This protein plays an important role in mitophagy, and mitochondrial fission and fusion is played out through its interaction with the system Parkin/PINK1.

The loss of function of Parkin, PINK1, and Omi/HtrA2 produces similar mitochondrial alterations in the *Drosophila melanogaster* and mammalian models. This could be understood as a clue to the participation of all of these factors in a common way being implicated in mitochondrial protection, but this appears to be a very complex scenario and it is not yet fully understood.

Omi/HtrA2, together with PINK1 and Parkin, seems to play a role in promoting mitochondrial integrity maintenance. In fact, Omi/HtrA2 can phosphorylate PINK1 at the mitochondria, after its own indirect phosphorylation. After this process, PINK1 protease activity is enhanced and therefore there is an increment in cell survival against oxidative stress (Plun-Favreau *et al.*, 2007). Moreover, in *D. melanogaster* models a genetic interaction has been observed between PINK1 and Omi/HtrA2, and overexpression of this protein can reverse phenotypic effects secondary to the loss of PINK1 activity, while PINK1 is not able to reverse degeneration in Omi/HtrA2.

Moreover, Omi/HtrA2 is able to activate autophagy by itself. Omi/HtrA2 participates actively in autophagy, by activating it through digestion of Hax-1, a Bcl-2 family-related protein that represses autophagy in a pathway dependent on Beclin 1. In fact, in neurodegenerative processes, mutations in Omi/HtrA2 are so deleterious because there is an inhibition of the autophagy induced by this protein, and thus the degradation of some neurodegenerative proteins is not possible, such as α-synuclein or polyglutamine-expanded huntingtin, or the endogenous autophagic substrate p62.

SPORADIC PD

The vast majority of patients with PD do not carry rare familial mutations. Epidemiological studies have noted increased risks of developing Parkinson's disease with

rural living, farming, drinking well water, and exposure to pesticides. Several occupations are high risk. Welding is associated with an increased risk versus the general population of roughly a factor of 10. The prevalence of Parkinsonism is about 1% in welders aged 40–69 years. Cabinet makers and cleaners are also high risk (Racette *et al.*, 2005).

In sporadic PD, altered or damaged mitochondrial proteins and impaired OXPHOS function are commonly observed in the substantia nigra. A stable decrease in complex I activity, increased ROS production, proton leak, and decreased maximum OXPHOS capacity also occur in PD cybrids (hybrid cells which contain the nuclear genome from a control and the mitochondrial genome from sporadic PD patients). All of these features are consistent with the involvement of OXPHOS dysfunction in sporadic PD, particularly with an emphasis on complex I. In fact we used complex I inhibitors as pharmacological tools to induce PD in experimental models. So, most of the pesticides (e.g., rotenone, paraquat) are complex I inhibitors and cause Parkinsonism symptoms (Dawson and Dawson, 2003). Furthermore, the mitochondrial toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine is another complex I inhibitor causing Parkinsonism in humans and rodents (Beal, 2001). The above inhibitors that cause PD symptoms in mice and humans induce mitochondrial fission.

Interestingly, several studies indicate that cell cycle proteins such as cyclin-dependent kinases (Cdks) are upregulated in neurodegenerative disease. For example, there is increased expression of cell cycle regulators in brain samples of PD and AD. In addition, MPTP, a neurotoxin that causes PD-like symptoms, induces a significant increase in Cdk2 and Cdk4 expression in CGNs. Furthermore, thrombin, a serine protease associated with AD symptomatology, induces Cdk4 activity in cortical neurons. Importantly, a broad-spectrum Cdk inhibitor (FLAV) and Cdk4 inhibitors protect neurons from MPTP- and thrombin-induced cell death, respectively.

More specifically, SH-SY5Y cell cultures showed a significant increase in the percentage of cells with either truncated or fragmented mitochondria after 6-OHDA treatment. This process was dependent on the mitochondrial fission factor Drp1. We observed that 6-OHDA recruited Drp1 to mitochondria. The relevance of this protein for 6-OHDA-induced mitochondrial fission was evidenced further by pretreating cells with mdivi-1. Consistent with these findings, we observed that mdivi-1 significantly attenuated 6-OHDA-induced mitochondrial fission.

Results from our laboratory showed that autophagy is induced by treatment with 6-OHDA (Solesio *et al.*, 2012). The 6-OHDA-induced autophagy correlated with an increase in the microtubule-associated protein 1A/1B-light chain 3 protein level, and with the accumulation of autophagic vacuoles in the cytoplasm. In 6-OHDA-challenged SH-SY5Y cells, the formation of autophagosomes took place away from the mitochondria, and in a time-dependent fashion.

DJ-1 overexpression in astrocytes is a way for the brain to try to protect itself against PD progression. DJ-1 upregulation in astrocytes represents a mechanism to protect themselves and neighboring neurons against environmental complex I toxicants, such as rotenone or paraquat. In fact, when there is a deficiency in DJ-1, there is an increment in the specific neurotoxicity induced by different specific inhibitors of this CTE complex. This effect was not observed when the inhibited were other complexes from the CTE. This neuroprotective effect of DJ-1 against CTE complex I inhibitors depends on proper astrocytic mitochondrial functioning. Thus, cells with deficiency in DJ-1 would impair mitochondrial motility,

fission-fusion cycles, membrane potential maintenance, and respiration in this kind of cell, both in basal levels and after inhibition of complex I. In one published work, apart from this fact, it has been demonstrated that the physical presence of neurons can affect astrocyte mitochondrial behavior.

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CHAPTER

15

Mitophagy Controlled by the PINK1-Parkin Pathway Is Associated with Parkinson's Disease Pathogenesis

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OUTLINE

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Abstract

Mitochondrial quality control is an important issue in higher animals that depends on aerobic respiration. A series of elegant studies have demonstrated that two Parkinson's disease-associated genes *PINK1* and *parkin* are involved in the maintenance of healthy mitochondria. Parkin in cooperation with PINK1 specifically recognizes damaged mitochondria with reduced mitochondrial membrane potential ($\Delta\psi m$), rapidly isolates them from the mitochondrial network, and eliminates them through the ubiquitin-proteasome and autophagy pathways. Here we introduce and review recent studies that contribute to understanding the molecular mechanisms of PINK1 and Parkin-mediated mitochondrial regulation. We also discuss how defects in the PINK1-Parkin pathway may cause neurodegeneration in Parkinson's disease.

INTRODUCTION

While eukaryotic cells have acquired the highly efficient power-generating system of aerobic respiration by incorporating mitochondria into the cytosol, alleviation of oxidative stress by appropriate regulation of mitochondria has become an emerging and inevitable issue. Nondividing cells or tissues with high energy demands in long-lived animals have particular need to regulate mitochondrial activity to avoid the production of deleterious reactive oxygen species. Mitochondrial dysregulation is now implicated in various human diseases including cancer, diabetes, myopathy, and neurodegeneration.

The pathological hallmark of the second most common neurodegenerative disorder Parkinson's disease (PD) is the progressive degeneration of dopaminergic neurons in the midbrain. Although mitochondrial dysfunction has long been believed to be closely associated with the etiology of PD, there was no direct evidence for the association. A series of studies on two PD-associated genes *PINK1* and *parkin* have now shown that these two genes work in a coordinated manner for mitochondrial maintenance including mitophagy of damaged mitochondria, strongly implying that dysregulation of mitochondria is one of the major causative or contributing factors in the etiology of PD.

PARKIN HAS UBIQUITIN LIGASE ACTIVITY

Parkin was originally identified as the gene associated with a familial form of PD known as autosomal recessive young-onset parkinsonism (AR-JP) linked to the *PARK2* locus, the major cause of young-onset PD (Kitada *et al.*, 1998). The gene product contains a ubiquitin-like (Ubl) domain at the N-terminus and two RING fingers flanking a cysteine-rich domain, termed in-between RING fingers (IBR), with ubiquitin ligase (E3) activity (Imai *et al.*, 2000; Shimura *et al.*, 2000) (Figure 15.1). There are two major types of E3: homologous to the E6AP carboxyl terminus (HECT)-type and RING finger-type E3; these function independently or form a multi-subunit complex. HECT-type E3s form a thioester intermediate of ubiquitin with a catalytic residue of cysteine before transferring the ubiquitin to the substrate, whereas RING finger-type E3s mediate the direct transfer of ubiquitin from E2 to the substrate. The RING-in-between-RING (RBR)-containing E3 family proteins have recently been proposed to possess hybrid E3 activity with properties of both HECT-type and RING finger-type E3s (Lazarou *et al.*, 2013). The Ubl domain has been shown to autoinhibit the

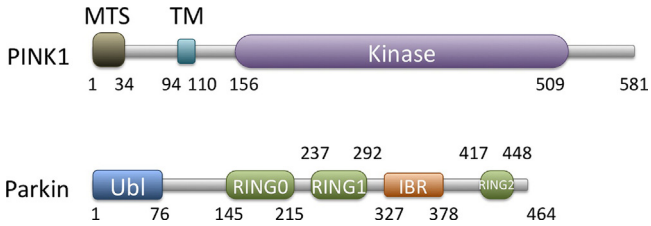

FIGURE 15.1 Parkin and PINK1 proteins. RING0 has been characterized as a new domain with similarity to conventional RING1 and RING2 domains (Hristova *et al.*, 2009). The numbers correspond to the residue numbers at the boundaries of the indicated domains. Abbreviations: MTS, mitochondrial targeting sequence; TM, transmembrane domain; Ubl, ubiquitin-like domain; RING, Ring finger motif; IBR, in-between RING fingers domain.

C-terminal RBR-containing region intramolecularly (Chaugule *et al.*, 2011), suggesting that Parkin is an inert E3 under normal conditions. Although several *Parkin*-deficient mouse lines have been generated and extensively examined as *Parkin*-linked PD models, most of them do not fully recapitulate dopaminergic neurodegeneration, hindering the determination of the pathophysiological role of the Parkin protein *in vivo*.

GENETIC ASSOCIATION BETWEEN THE PD GENES *PARKIN* AND *PINK1*

The *PTEN-induced putative kinase 1* (*PINK1*) gene, which encodes a serine-threonine kinase with a mitochondrial targeting signal at the N-terminus (Figure 15.1), was identified as a gene associated with another recessive young-onset form of familial PD linked to *PARK6*. Although its clinical and pathological features closely resemble those of *PARK2*, *PINK1*-deficient mice do not show the loss of dopaminergic neurons in the midbrain and its physiological functions remain unclear. In 2003, Greene *et al.* (2003) reported that loss of the *Parkin* gene in *Drosophila* resulted in massive degeneration of muscle tissues and a defect in spermatogenesis caused by mitochondrial degeneration (Greene *et al.*, 2003). Similar mitochondrial phenotypes were found in *PINK1*-deficient or knockdown *Drosophila*. Genetic epistasis studies have suggested that *PINK1* is upstream of *Parkin* and that these two genes are indispensable for the maintenance of mitochondrial functions in *Drosophila* (Clark *et al.*, 2006; Park *et al.*, 2006; Yang *et al.*, 2006).

PINK1 AND PARKIN ARE INVOLVED IN THE REGULATION OF MITOCHONDRIAL DYNAMICS

A phenotypic feature produced by the loss of *PINK1* in *Drosophila* is the accumulation of elongated or aggregated mitochondria in the flight muscles and central dopaminergic neurons, a character that is closely associated with mitochondrial degeneration (Deng *et al.*, 2008; Yang *et al.*, 2008). These mitochondrial defects cause age-dependent motor impairment and decreased sperm fertility. A similar morphological abnormality has been observed in

Parkin-deficient flies, in which highly fused and swollen mitochondria with indistinct cristae were observed (Deng *et al.*, 2008; Imai *et al.*, 2010). *PINK1* and *Parkin* mutant phenotypes are partly rescued by increased activity of Drp1, which is a component of the mitochondrial fission machinery, or by the reduced activity of mitofusin (Mfn) or optic atrophy 1 (OPA1), which are involved in mitochondrial fusion events (Deng *et al.*, 2008; Yang *et al.*, 2008). Abnormal mitochondrial morphology and dynamics are also observed in mammalian cultured cells and hippocampal and dopaminergic neurons (Yang *et al.*, 2008). These findings suggest that PINK1 and Parkin are involved in the positive regulation of mitochondrial fission and the maintenance of mitochondrial functions in both insects and mammals.

PARKIN IS INVOLVED IN THE ELIMINATION OF DAMAGED MITOCHONDRIA THROUGH THE MITOPHAGY PATHWAY

Parkin is mainly localized in the cytosol when expressed in cultured cells, but it remains unknown how Parkin regulates the function of mitochondria. Narendra *et al.* (2008) reported that when $\Delta\psi m$ of cultured mammalian cells is disrupted by mitochondria-damaging reagents such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), Parkin is translocated to mitochondria with low $\Delta\psi m$ (Narendra *et al.*, 2008). This translocation induces LC3-mediated autophagic elimination of the damaged mitochondria called mitophagy (Narendra *et al.*, 2008) (Figure 15.2). The mitochondrial accumulation of proteins that are polyubiquitinated mainly with Lys63-linked polyubiquitin and a small proportion of Lys48 linkages (Chan *et al.*, 2011; Lee *et al.*, 2010) recruits the ubiquitin- and LC3-binding adaptor protein p62/SQSTM1 (Geisler *et al.*, 2010; Narendra *et al.*, 2010a) and ubiquitin-binding deacetylase HDAC6 (Lee *et al.*, 2010) after Parkin translocation (Figure 15.3). Although the details of this mechanism are unresolved, Lys63-linked polyubiquitination may contribute to the proteasomal degradation of mitochondrial proteins and the HDAC6- and/or p62-mediated sequestration of mitochondria (Lee *et al.*, 2010) (Figure 15.3). Depolarized mitochondria treated with CCCP or paraquat accumulate in the perinuclear compartment in a p62/SQSTM1-dependent manner (Geisler *et al.*, 2010; Narendra *et al.*, 2010a), which is followed by the activation of autophagic isolation and subsequent lysosomal degradation of the mitochondria (Narendra *et al.*, 2008) (Figure 15.2). The clustering of ubiquitinated mitochondria by p62 and HDAC6 is reminiscent of the sequestration of ubiquitinated proteins called aggresomes, which may facilitate isolation of the damaged mitochondria from the healthy mitochondrial network and their efficient removal. The role of p62/SQSTM1 in mitophagy will be discussed later.

PINK1 REGULATES THE MITOCHONDRIAL TRANSLOCATION OF PARKIN

The translocation of Parkin from the cytosol to the mitochondria, which requires intact PINK1 with kinase activity, is an initial step in the mitophagy process in mammalian (Geisler *et al.*, 2010; Kawajiri *et al.*, 2010; Matsuda *et al.*, 2010; Narendra *et al.*, 2010b; Vives-Bauza *et al.*, 2010) and *Drosophila* cultured cells (Ziviani *et al.*, 2010) (Figure 15.2). Most of the

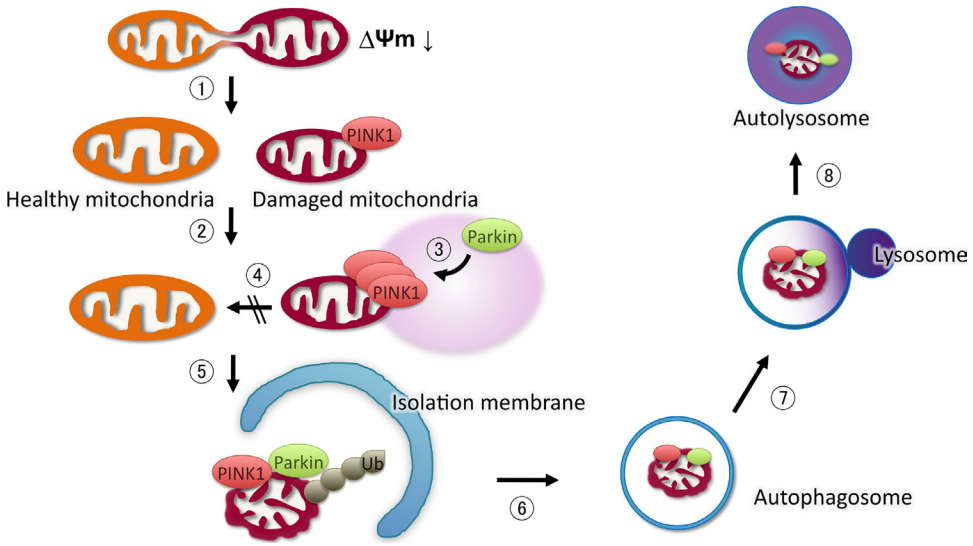

FIGURE 15.2 Mitophagy by the PINK1-Parkin pathway. Fusion/fission is required for the maintenance of a healthy mitochondrial population. Mitochondrial fusion is believed to require the exchange of a set of internal components, including copies of the mitochondrial genome, respiratory proteins, and metabolic products. Mitochondrial fission may play a role in the removal of damaged mitochondria (1, 2) with a reduced Δψm through an autophagy-lysosomal pathway, i.e., "mitophagy." PINK1 is constitutively degraded in healthy mitochondria. Following a decrease in Δψm, PINK1 is stabilized on the mitochondrial outer membrane (OM) (3). The accumulation of PINK1 induces the translocation of Parkin from the cytosol to the mitochondria (3), which leads to Parkin-dependent mitochondrial protein degradation through which Mfn is degraded to prevent healthy mitochondria from re-fusing with damaged mitochondria (4). Subsequently, Parkin activates the autophagy machinery that includes induction of the isolation membrane for autophagy (5). Mitochondria encompassed by the isolation membrane are degraded by a conventional autophagy reaction (6–8).

pathogenic mutations found in PINK1 and Parkin compromise the mitochondrial translocation activity of Parkin, which was partly confirmed using a neuronal culture derived from iPS cells obtained from *PINK1*-linked PD cases (Seibler *et al.*, 2011). Thus, Parkin-mediated mitophagy may be closely associated with the etiology of young-onset PD caused by *PINK1* and *Parkin* mutations.

The E3 activity of Parkin is activated after mitochondrial translocation in a PINK1-dependent mechanism, where PINK1-dependent phosphorylation of Parkin Ser65 in the Ubl domain and oligomerization of Parkin may be involved (Kondapalli *et al.*, 2012; Lazarou *et al.*, 2013; Shiba-Fukushima *et al.*, 2012). Activated Parkin degrades several proteins localized in the mitochondrial outer membrane (OM) through the ubiquitin-proteasome pathway, which includes Mfn1 and Mfn2, Drp1, voltage-dependent anion channel 1 (VDAC1), Miro, mitoNEET, and hexokinase I. Although there appear to be no common motifs for recognition by Parkin in these proteins, degradation of the mitochondrial elongation factor Mfn by Parkin, also observed in *Drosophila* cultured cells, contributes to the fragmentation of the mitochondria during mitophagy (Ziviani *et al.*, 2010). This finding is consistent with observations in *Drosophila*, in which the loss of *PINK1* or *Parkin* leads to

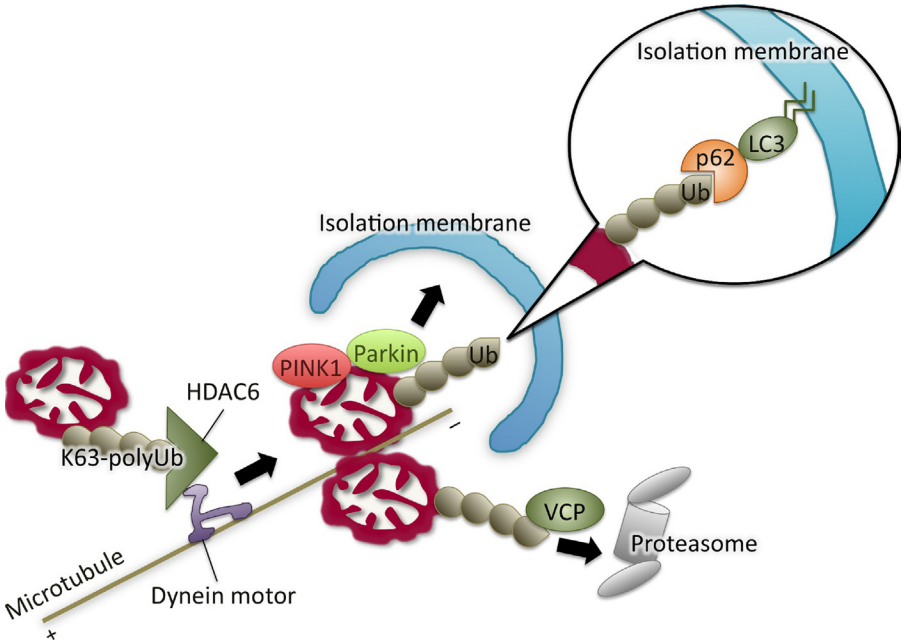

FIGURE 15.3 Proteins involved in mitophagy mediated by PINK1-Parkin. Parkin activation stimulates Lys63-linked polyubiquitination of mitochondrial OM proteins, which is recognized by HDAC6. The HDAC6-dynein complex transports the polyubiquitinated mitochondria to the perinuclear region along microtubules. The polyubiquitinated mitochondrial proteins are also recognized by either the proteasome or p62. The proteasome degrades ubiquitinated OM proteins such as Mfn and Miro in cooperation with VCP/p97, whereas p62 recruits the LC3-mediated autophagy machinery to remove the damaged mitochondria.

mitochondrial elongation and a reduction of Mfn activity that partly rescues the mitochondrial degeneration (Deng *et al.*, 2008; Yang *et al.*, 2008). However, the elimination of Mfn by Parkin and the perinuclear aggregation of mitochondria by p62/SQSTM1 *per se* appear to be dispensable for mitophagy in mammalian cells (Chan *et al.*, 2011; Narendra *et al.*, 2010a), although the requirement for p62 is controversial (Geisler *et al.*, 2010). It is very likely that a p62-related protein NBR1 is also recruited to the damaged mitochondria in cultured mammalian cells. Thus, NBR1 may compensate the p62 function (Chan *et al.*, 2011). Mfn degradation and mitochondrial perinuclear clustering may prevent the re-fusion of damaged mitochondria with the healthy mitochondrial network and the axonal transportation of damaged mitochondria. In addition, mitochondrial reorganization by the inactivation of Mfn and recruitment of p62 may facilitate the isolation of mitochondria by autophagosomes.

MOLECULAR REGULATION OF PINK1 AND PARKIN

PINK1 was originally isolated as one of the genes induced by the tumor suppressor PTEN, suggesting that the PI3K/AKT pathway negatively regulates *PINK1* expression.

Later, FoxO, a downstream component of the PI3K/AKT pathway, was shown to transactivate the mammalian *PINK1* promoter (Mei *et al.*, 2009) Although *PINK1* transcripts are abundantly expressed in cultured cells and several mitochondria-rich tissues including the heart, skeletal muscle and testis, it is difficult to detect endogenous PINK1 protein, which has made it challenging to elucidate the molecular action of PINK1. However, PINK1 was found to accumulate rapidly in depolarized mitochondria (Matsuda *et al.*, 2010; Narendra *et al.*, 2010b; Vives-Bauza *et al.*, 2010), suggesting that PINK1 is subject to post-translational degradation. Several studies report that the rhomboid family protease presenilin-associated rhomboid-like protein (PARL), which is localized in the mitochondrial inner membrane (IM), processes PINK1 in a $\Delta\psi$m-dependent manner (Jin *et al.*, 2010). PINK1 that is newly synthesized in the cytosol is inserted into the IM through the mitochondrial import activity of the Tom complex (Lazarou *et al.*, 2012) and is cleaved into its putative transmembrane domain by PARL to generate the 52-kDa form of PINK1. The 52-kDa form is rapidly removed by a proteasome-dependent pathway, most likely after its release from the mitochondrial intermembrane space (IMS) to the cytosol (Jin *et al.*, 2010).

Upon depolarization of $\Delta\psi$m, IM insertion and the subsequent processing of PINK1 by PARL may be inhibited, leading to the accumulation of full-length PINK1 in the mitochondrial OM, most likely facing the cytosol (Jin *et al.*, 2010). However, it remains controversial whether the processing of PINK1 by PARL is required for Parkin recruitment upon depolarization of the mitochondria, and further studies will be necessary to completely resolve the topological changes of the processed forms of PINK1 (Jin *et al.*, 2010).

The accumulation and autophosphorylation of PINK1 possibly stimulate its kinase activity that is required for the mitochondrial translocation of Parkin (Lazarou *et al.*, 2012). Although the detailed molecular mechanisms by which PINK1 recruits Parkin to mitochondria remain unclear, recent data suggest that PINK1-dependent phosphorylation of Parkin in the Ubl domain and Parkin self-association via the RBR region are involved (Lazarou *et al.*, 2013; Rakovic *et al.*, 2011; Shiba-Fukushima *et al.*, 2012). Moreover, the requirement of the E3 activity of Parkin is also indicated (Lazarou *et al.*, 2013). Phosphorylation of the Parkin Ubl domain by PINK1 may also contribute to the stimulation of Parkin E3 activity through release of the RBR region from the autoinhibitory mechanism by the Ubl domain (Kondapalli *et al.*, 2012) and/or may stimulate its self-association for mitochondrial translocation (Lazarou *et al.*, 2013).

Parkin is also regulated at the transcriptional level. The unfolded protein response (UPR) transactivates Parkin transcripts via ATF4, a transcription factor involved in the UPR (Bouman *et al.*, 2011; Imai *et al.*, 2000). Mitochondrial damage may induce activation of the UPR (Bouman *et al.*, 2011) and oxidative stress leading to the upregulation of Parkin expression. In other stress conditions, such as γ-irradiation or hydrogen peroxide, p53 transactivates Parkin (Zhang *et al.*, 2011). Conversely, Parkin is reported to suppress the transcription of p53 by direct binding to the *p53* promoter region, suggesting a negative feedback mechanism. The p53-dependent induction of *Parkin* is implicated in the regulation of glucose metabolism because the loss of *Parkin* activates glycolysis and reduces mitochondrial respiration, leading to the Warburg effect (Zhang *et al.*, 2011). This phenotype may be associated with the accumulating evidence from tumour tissues, cell lines, and model animals that Parkin functions as a tumor suppressor.

PINK1 AND PARKIN REGULATE MITOCHONDRIAL MOTILITY

Various mitochondrial proteins are ubiquitinated and degraded through the ubiquitin-proteasome pathway after the mitochondrial translocation of Parkin (Chan *et al.*, 2011). It is not clear whether all of these proteins are ubiquitinated specifically by Parkin. Among the mitochondrial proteins that are degraded during mitophagy, Mfn (see above) and Miro are well characterized as Parkin substrates. The Miro family proteins contain two GTPase domains separated by a linker region containing putative calcium-binding EF hand motifs. Miro is involved in microtubule-dependent mitochondrial transport through its binding partners Milton and kinesin heavy chain (KHC). Miro has been identified as one of the binding partners of PINK1 and was isolated as a gene that modulates the phenotypes of *PINK1* mutant flies (Liu *et al.*, 2012). Two recent reports have demonstrated that Miro is degraded through the PINK1–Parkin pathway, which aids in the removal of damaged mitochondria from mitochondrial trafficking pathways (Liu *et al.*, 2012; Wang *et al.*, 2011) (Figure 15.4). Wang *et al.* (2011) have also shown that phosphorylation of Miro by PINK1 is a prerequisite for its degradation via Parkin, although our study failed to observe Miro phosphorylation or the requirement of this modification for Miro degradation (Liu *et al.*, 2012).

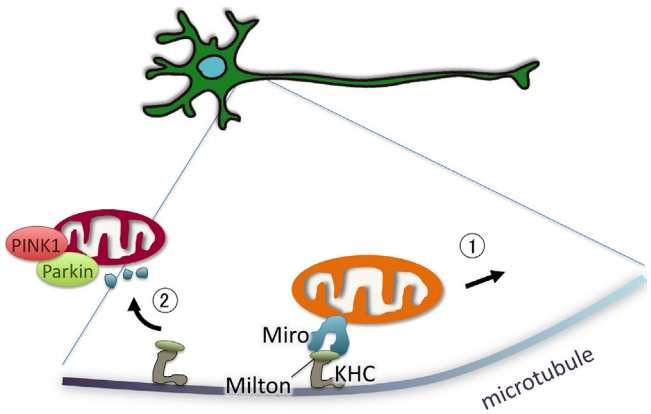

FIGURE 15.4 Regulation of mitochondrial motility by PINK1-Parkin. Quarantine control of mitochondrial transportation by PINK1-Parkin signaling. Mitochondria are transported along the microtubule network through the Miro-Milton-KHC complex (1). In neurons, the transportation of healthy mitochondria to the nerve terminal ensures a stable energy supply for a variety of synaptic activities. PINK1 is activated in mitochondria with reduced $\Delta\psi m$ and recruits Parkin, resulting in Miro degradation; thus, damaged mitochondria are selectively removed from the mitochondrial transport machinery (2). These mitochondria may be further eliminated by PINK1-Parkin-mediated mitophagy.

PHYSIOLOGICAL AND PATHOLOGICAL ROLES OF MITOPHAGY BY THE PINK1-Parkin PATHWAY

Although PINK1-Parkin signalling appears to be conserved, at least between mammals and *Drosophila*, *PINK1*- and *Parkin*-deficient mice do not recapitulate the symptoms of PD. The molecular mechanism underlying mitochondrial quality control by the PINK1-Parkin pathway has been mostly characterized in Parkin-transfected cultured cells using potent mitochondria-damaging reagents such as CCCP. Therefore, the relevance of mitophagy observed in the cultured cells to the etiology of PD remains debatable. A recent report has pointed out that endogenous levels of Parkin degrade substrates on the mitochondrial OM such as Mfn but cannot induce the autophagy reaction to remove whole mitochondria (Rakovic *et al.*, 2011). Parkin protects cells from various stresses including oxidative stress, endoplasmic reticulum (ER) stress, and mitochondrial stress. However, it has been reported that the mitophagy pathway is not integrated in the stress protection mechanism by Parkin (Muller-Rischart *et al.*, 2013). Nevertheless, the accumulating evidence of mitochondrial abnormalities in animal models and PD patients implies that mitochondrial maintenance by the PINK1-Parkin pathway is closely linked to the pathogenesis of the disease. Abnormal deletion of the mitochondrial genomes is frequently observed in the substantia nigra, one of the affected regions of PD. It has been empirically shown that mitochondrial populations harboring mutant mitochondrial genomes are selectively eliminated by Parkin (Suen *et al.*, 2010). The ubiquitination and elimination of Mfn does not occur in human fibroblasts derived from *PINK1*- and *Parkin*-linked PD patients following oxidative stress induction and depolarization of the mitochondria. Recent studies in *Parkin*- and *PINK1*-deficient mice have reported morphological and functional alterations of the mitochondria in neurons and astrocytes. Like the muscle degeneration in *Drosophila*, the function of the heart, which depends on highly developed cardiac muscle mitochondria, is also impaired with increased oxidative stress in *PINK1*-null mice. Ischemic preconditioning exerts cardioprotective effects in heart failure models in which the mitochondrial translocation of Parkin is induced. The deletion of *Parkin* in mice abolishes this effect. Although the role of PINK1 and Parkin in the cardiac function of humans is yet unknown, it is worth noting that the prevalence of heart failure in elderly PD patients is twice that in elderly individuals without PD.

A missense mutation of the mitophagy-associated *PARL* gene found in PD cases abolishes PINK1 processing activity and subsequent Parkin-mediated mitophagy. No polymorphisms in the *Miro* gene have been linked to PD to date, although the inactivation of Mfn2 but not Mfn1 in dopaminergic neurons of the mouse brain resulted in progressive degeneration of the nigrostriatal pathway, suggesting a functional difference between Mfn1 and Mfn2 in neurons. Mutations in Mfn2 cause Charcot-Marie-Tooth disease type 2A, a peripheral nerve disorder characterized by a slow progressive degeneration of the muscles of the extremities. Mfn2 has been shown to be required for axonal mitochondrial transport by direct binding to Miro. In this context, the mitochondrial fusion activity is suggested to be inessential for mitochondrial transport, because knockdown of OPA1, another protein for mitochondrial fusion, does not affect mitochondrial motility.

DISCUSSION

One of the neuropathological features of PD is the accumulation of protein inclusions called Lewy bodies, which implies a dysfunction of protein quality control regulated by the ubiquitin-proteasome and autophagy pathways. Some PD and PD-related gene products are characterized as components of the protein quality control system (e.g., LRRK2, ATP13A2, HtrA2, and FBXO7) or they are abnormal proteins themselves (α-synuclein, Tau). In fission yeast, impairment of the ubiquitin-proteasome pathway induces the accumulation of reactive oxygen species in mitochondria (Takeda *et al.*, 2010). These mitochondria are removed by the autophagy pathway (Takeda *et al.*, 2010). In a *Drosophila* model, it has been shown that mitochondria with accumulated misfolded proteins are also removed by Parkin. The phenomenon is reminiscent of ER-associated protein degradation (ERAD), which eliminates misfolded or unassembled proteins from the ER. An AAA+ ATPase p97/VCP, which is required for ERAD along with proteasome activity, is involved in the elimination of proteins on the OM during mitophagy (Kim *et al.*, 2013). Thus, the pathways for protein quality control and mitochondrial maintenance are likely to be linked to each other.

Two gene products implicated in young-onset PD, PINK1 and Parkin, have recently been implicated in mitochondrial quality control. PINK1–Parkin-mediated mitophagy includes two major components: commitment to elimination by PINK1 and Parkin and execution of elimination by autophagy. Although canonical autophagy components appear to be involved in the autophagy stage, at least under experimental conditions, the molecular mechanisms by which PINK1 and Parkin signal the commitment to elimination remain largely unclear. This is an important issue and its resolution may lead to the identification of new genes associated with neurodegenerative disorders and the understanding of the etiology of idiopathic PD as well as *PINK1*- and *parkin*-linked PD.

Acknowledgments

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CHAPTER

16

Loss of Mitochondria during Skeletal Muscle Atrophy

Sudarsanareddy Lokireddy

OUTLINE

Abstract

Much of our current understanding of skeletal muscle atrophy has come from studies on patients having lost significant muscle mass as a result of inactivity, nerve damage, aging (sarcopenia), or various systemic diseases including cancer, AIDS, heart failure, liver cirrhosis, kidney failure, sepsis, and diabetes. Current evidence has clearly demonstrated that loss of muscle mass and function results largely from accelerated protein degradation by the ubiquitin-proteasome system (UPS) and autophagy-lysosomal system (ALS). In recent years, there has been great progress in defining the signaling mechanisms behind increased protein degradation during muscle atrophy from a genetic perspective. However, many open questions remain regarding the cascade of events that give rise to the diverse and complex muscle pathology. Recent discoveries highlight the importance of mitochondria loss during muscle atrophy because of the large energy requirements to maintain muscle function. Mitochondria are dynamic organelles whose function and morphology are controlled by fusion and fission events. During muscle atrophy, both morphology and function of mitochondria undergo dramatic changes. Furthermore, dysregulation of mitochondrial dynamics during muscle atrophy affects many signaling pathways, which can further exacerbate atrophy.

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INTRODUCTION

Skeletal muscle is a type of contractile tissue found in vertebrates, and it is derived from the mesodermal layer of embryonic germ cells. Skeletal muscle is one of the largest organs in the human body, comprising about 40% of the total body mass. In normal physiological conditions, the maintenance of muscle mass and strength depends on the balance between anabolic and catabolic events (Mitch and Goldberg, 1996) (Figure 16.1). Dysregulation of metabolic reactions occurring in muscle often results in atrophy or cachexia. Muscle atrophy is a highly debilitating condition occurring as a result of various pathological states such as cancer, AIDS, liver cirrhosis, kidney failure, heart failure, sepsis, obesity, disuse, aging, and diabetes. The adverse consequences of muscle atrophy are not limited to a decrease in muscle function, but can profoundly affect overall quality of life of patients (Fearon *et al.*, 2011). Cachexia is clinically distinct from anorexia and is defined as a complex metabolic syndrome characterized by loss of muscle with or without loss of fat mass, which cannot be prevented through nutritional intervention alone (Fearon *et al.*, 2011). Specifically,

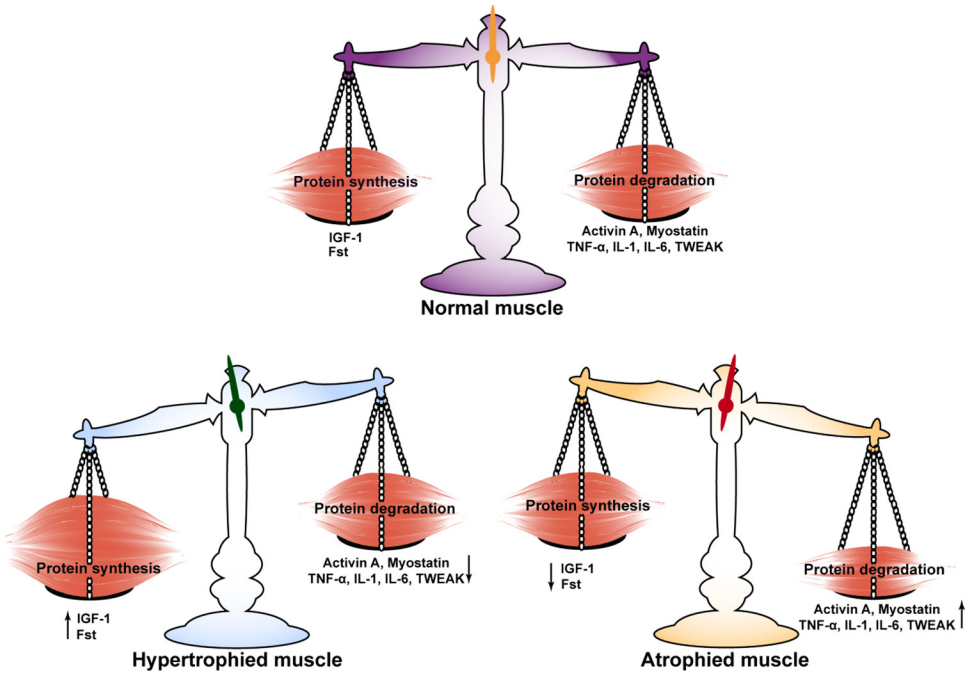

FIGURE 16.1 Schematic diagrams representing the protein metabolisms (protein synthesis and degradation) in normal, hypertrophied, and atrophied muscle. Evidence clearly supports that loss of muscle mass and function results largely from accelerated protein degradation by the ubiquitin-proteasome system (UPS) and autophagy-lysosomal system (ALS) in atrophied muscle stimulated by growth factors (activin A and myostatin) and cytokines (TNF-α, IL-1, IL-6 and TWEAK). Conversely, it has also been shown that reduced levels of IGF-1 and Fst in circulation results in suppression of protein synthesis during muscle atrophy. In support of that, overexpression of either IGF-1 or Fst leads to hypertrophied muscle due to increased protein synthesis and reduced protein degradation.

cancer-associated loss of muscle, termed cancer cachexia, is documented in over half of cancer patients, and its onset often leads to a poor prognosis (Fearon *et al.*, 2011). One third of cancer-related deaths can be attributed to cancer-associated cachexia rather than the cancer itself (Fearon *et al.*, 2011).

Extracellular growth regulators secreted by muscle, called myokines, manipulate muscle development and growth by altering the transcriptional program, protein synthesis, protein degradation, and cellular fate (Pedersen and Febbraio, 2012). Cancer-induced muscle atrophy mainly occurs due to high levels of circulating humoral and tumoral signaling factors. Specifically, cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), and TNF-related weak inducer of apoptosis (TWEAK) have been shown to induce muscle atrophy through promoting protein degradation and suppressing protein synthesis (Fearon *et al.*, 2012) (Figure 16.1). Recently several growth factors, namely activin A, myostatin, IGF-1, and follistatin, secreted into the extracellular milieu of muscles, have been shown to modulate survival, proliferation, and differentiation of muscle cells (Fearon *et al.*, 2012). Interestingly, mutations or deletion in the myostatin gene in human, mice, and certain cattle breeds (Belgian bull), result in an increase in both the number and size of the muscle fibers, resulting in the development of what is termed a "double-muscled" phenotype (Schuelke *et al.*, 2004). In addition to the inhibitory roles that activin A and myostatin play during skeletal muscle myogenesis, a plethora of evidence now implicates activin A and myostatin in the induction of skeletal muscle atrophy. Increased activin A and myostatin expression has been detected in atrophic conditions such as muscle disuse, including chronic human disuse atrophy, hind limb unloading and space flight, cancer cachexia, food deprivation, and denervation (Lokireddy *et al.*, 2012a; Zhou *et al.*, 2010). Mechanistically, activin A and myostatin induce the atrophy by activating the ubiquitin-proteasome system (UPS) and autophagy-lysosomal system (ALS) and thus promoting the degradation of muscle proteins (Lokireddy *et al.*, 2012c; Zhou *et al.*, 2010). Conversely, it has also been demonstrated that the severe body mass reduction resulting from myostatin overexpression was ameliorated through the injection of myostatin propeptide (LAP) or follistatin into mice, two well-characterized antagonists of myostatin and activin A signaling (Fearon *et al.*, 2012) (Figure 16.1). In addition, inactivating activin A and myostatin by soluble activin receptor type IIB (sActRIIB) not only reduced the activity of the ubiquitin-proteasome pathway but also ameliorated skeletal muscle atrophy induced by the C26 tumor. In addition to follistatin, an important insulin-like growth factor-1 (IGF-1) is a critical regulator of muscle hypertrophy (which is accompanied by an increase in IGF-1 expression in muscle), repair, and maintenance (Glass, 2005). Moreover, IGF-1 treatment enhances protein synthesis in muscle through the activation of the PI3K/Akt pathway (Glass, 2005) (Figure 16.1). Treatment with IGF-1 also suppresses protein degradation by phosphorylation of the FoxO1/3 transcription factor, a master regulator of muscle atrophy program by pAkt (Fearon *et al.*, 2012) (Figure 16.1). Further details regarding the mechanisms of muscle atrophy will be discussed extensively in the next section.

Mitochondria are small, independently replicating organelles that play a major role in aerobic energy metabolism by generating the bulk of cellular ATP, the primary energy currency of the cell. Mitochondria are particularly important for muscle function because of the large energy requirements of this organ. Defects in mitochondrial function in muscle result in a group of diseases termed mitochondrial myopathies. Mitochondrial myopathies

are a group of complex neuromuscular disorders and can occur as a result of other diseases such as cancer, diabetes, and obesity, or even during aging, especially sarcopenia (Holt *et al.*, 1988; Vafai and Mootha, 2012). Sarcopenia is defined as the unintentional loss of muscle mass and strength associated with aging. Approximately 25% of adults above 45 years lose 5% of muscle mass per decade from the second half of the fifth decade onwards. Sarcopenia is characterized by a decrease in muscle fiber number and size during aging. Recent reports have demonstrated that fiber type switching (from fast to slow), reduced activation of satellite cells (muscle stem cells), and increasing oxidative stress with decreased mitochondrial activity are characteristic features of sarcopenic muscle loss. Studies on muscle specimens from healthy individuals have revealed age-related declines in mitochondrial mass, activities of tricarboxylic acid (TCA) cycle enzymes, O_2 consumption, and ATP synthesis after the age of 50. Moreover, a 40–50% decrease in oxidative phosphorylation (OXPHOS) activity has also been detected.

Individuals afflicted with mitochondrial disorders demonstrate muscular anomalies such as asthenia as well as breathlessness, exercise intolerance, heart failure, dementia, stroke-like symptoms, deafness, blindness, seizures, heavy eyelids, and vomiting (Vafai and Mootha, 2012). Originally, mitochondrial myopathies were diagnosed based solely on clinical findings. In most situations, mitochondrial myopathies result from mutations in mitochondrial proteins that modulate the structure and function of the organelle (Holt *et al.*, 1988). Generally, mutation of mitochondrial proteins encoded in the nucleus results in clinical symptoms that develop during early childhood, while mtDNA (mitochondrial DNA) mutations lead to clinical manifestations that develop in late childhood or early adulthood (Holt *et al.*, 1988; Vafai and Mootha, 2012). In some instances, significant reduction in the number of mitochondria is observed in muscle during mitochondrial myopathies and muscle atrophy (Vafai and Mootha, 2012). Mitochondrial loss during muscle atrophy will be discussed extensively in the next section.

UBIQUITIN-PROTEASOME SYSTEM MEDIATED MUSCLE ATROPHY

The ubiquitin-proteasome system (UPS) is the major proteolytic system in mammalian cells and plays an important role during muscle atrophy (Mitch and Goldberg, 1996). UPS-mediated proteolysis is an ordered process that involves polyubiquitination of substrates and their subsequent degradation by 26S proteasomes (Mitch and Goldberg, 1996). During ubiquitination, ubiquitin is first activated by a ubiquitin-activating enzyme (E1), which catalyzes a two-step ATP-dependent process resulting in the formation of a ubiquitin-E1 thiol ester product (Jagoe and Goldberg, 2001). Activated ubiquitin is transferred to a ubiquitin-conjugating enzyme (E2), also termed ubiquitin carrier protein. The ubiquitin-conjugated E2 enzyme then associates with a ubiquitin ligase (E3), which is responsible for the transfer of ubiquitin from the E2 enzyme to a lysine side chain on a specific protein substrate, forming an isopeptide bond. Ubiquitin itself carries seven lysines, which can be used as acceptor residues in subsequent rounds of ubiquitin conjugation, resulting in poly-ubiquitination. In some circumstances, a fourth enzyme (E4) functions to lengthen the ubiquitin chains on target proteins. Finally, the ubiquitin-tagged proteins are targeted for degradation by the 26S proteasomes (Jagoe and Goldberg, 2001; Mitch and Goldberg, 1996).

Muscle atrophy results in upregulation of several E3 ligases called atrogenes (atrophy-related genes), including the muscle-specific enzymes atrogin-1 (MAFbx) and MuRF1 (Glass, 2005). Expression of both atrogin-1 and MuRF1 is consistently induced in several forms of muscle atrophy associated with various cancers, immobilization, denervation, hindlimb suspension, glucocorticoid (dexamethasone) treatment, and addition of the cachectic cytokines, TNF-α, IL-1, IL-6, and TWEAK, and growth factors myostatin and activin A (Fearon *et al*., 2012; Zhou *et al*., 2010). Mice deficient in atrogin-1 or MuRF1 are more resistant to the effects of denervation- and fasting-induced muscle atrophy (Glass, 2005). Current literature suggests that MuRF1 specifically targets and degrades sarcomeric proteins, including myosin heavy chain (Myh) and myosin light chain (Myl), whereas atrogin-1 ubiquitinates MyoD, a promyogenic factor, and eukaryotic translation initiation factor 3 subunit F (eIF3-f), a critical component in RNA translation machinery. In addition, desmin is also a target of atrogin-1 during myostatin-induced muscle atrophy in muscle culture models. Myostatin-induced skeletal muscle atrophy results in the downregulation of myogenic gene expression (Lokireddy *et al*., 2012b). Overexpression of myostatin in postnatal skeletal muscle reduced the expression of several myogenic structural genes, including Myh and desmin. Furthermore, myostatin-mediated muscle atrophy resulted in the depression of key myogenic regulatory factors, including MyoD and myogenin. A reduction in these key myogenic genes would serve to exacerbate the atrophy phenotype by potentially impairing postnatal myogenesis and muscle regeneration. Concomitant with the downregulation of key genes involved with myogenesis, myostatin-mediated muscle atrophy results in the upregulation of genes involved with the UPS including atrogin-1 and MuRF1 (Lokireddy *et al*., 2011). In the same study it was demonstrated that treatment of myotubes with recombinant myostatin protein antagonized the IGF-1/PI3K/Akt pathway, resulting in the enhanced activation of the transcription factor FoxO1/3 and subsequent activation of atrophy-related genes *atrogin-1* and *MuRF1* (Lokireddy *et al*., 2011).

In atrophying muscle, reduced protein levels of PGC-1α (a master regulator for mitochondrial biogenesis) were observed during the fasting- and denervation-induced muscle atrophy (Sandri *et al*., 2006). Overexpression of PGC-1α in muscle negatively regulates FoxO3a transcription factor activity and suppresses the activation of FoxO3 in atrophying muscle (Sandri *et al*., 2006). Furthermore, the PGC-1α isoform PGC-1α4 was shown to regulate expression of growth factors such as IGF-1, and myostatin was shown to promote the muscle hypertrophy during chronic exercise in humans (Ruas *et al*., 2012).

Recently, another E3 ligase, Trim32, which ubiquitously expressed in all tissues, promotes the degradation of thin filaments, Z-bands, and the desmin cytoskeleton during fasting-induced muscle atrophy (Cohen *et al*., 2012). Downregulation of Trim32 during fasting reduced fiber atrophy and the rapid loss of thin filaments. Furthermore, Trim32 has been shown to target degradation of desmin filaments upon fasting, which was accompanied by increased phosphorylation of desmin (Cohen *et al*., 2012). Interestingly, another Fbxo class E3 ligase, Fbxo40, may contribute to muscle atrophy by inducing the ubiquitination of IRS1 and thus limiting the IGF-1/PI3K/Akt pathways and downstream protein synthesis activation (Shi *et al*., 2011). Fbxo40 has been shown to mediate IRS1 degradation through the UPS mechanism upon its phosphorylation by IGF1R and IGF-1 (Shi *et al*., 2011).

The NF-κB pathway has also been implicated in skeletal muscle atrophy. Activation of the classical NF-κB pathway by pro-inflammatory cytokines, such as TNF-α, IL-1, and IL-6,

promotes skeletal muscle atrophy (Fearon *et al.*, 2012). Supportingly, transgenic mice with enhanced NF-κB activity show a dramatic muscle atrophy phenotype. Profound muscle atrophy in these mice was associated with an increase in the expression of MuRF1 (Cai *et al.*, 2004). The activation of the NF-κB pathway requires the UPS-mediated degradation of the inhibitor of kappa B (IκB) protein, which binds to NF-κB and represses its nuclear import and thus transcriptional activity. TNF-α-mediated NF-κB activation resulted in the inhibition of MyoD and consequently impaired skeletal muscle differentiation. Further studies of the molecular action of TNF-α revealed that the cytokine promotes ubiquitin-mediated degradation of the NF-κB inhibitor, IκBα, resulting in enhanced NF-κB signaling (Guttridge *et al.*, 2000).

Several studies have also demonstrated that TNF-α-mediated NF-κB signaling results in enhanced UPS activity (Fearon *et al.*, 2012). Administration of TNF-α was shown to increase the levels of ubiquitin-conjugated proteins. Conversely, inhibition of TNF-α through injection of anti-TNF-α antibodies resulted in a decrease in ubiquitinated proteins in AH-130 tumor-bearing rats. It has also been demonstrated that the addition of TNF-α promoted the upregulation of atrogin-1 in cultured muscle cells. Treatment with TWEAK resulted in activation of the NF-κB signaling cascade and the inhibition of myoblast differentiation (Fearon *et al.*, 2012). Furthermore, TWEAK inhibited the expression of MyoD and myogenin, and also promoted MyoD degradation. Therefore, TWEAK negatively regulates myogenesis through the activation of NF-κB and degradation of MyoD (Fearon *et al.*, 2012). In addition to TNF-α and TWEAK, another cytokine, IL-1, has been associated with diminished food intake and subsequent induction of anorexia (Fearon *et al.*, 2012). However, the role of IL-1 in cachexia is a matter of dispute. Injection of an IL-1 receptor antagonist failed to ameliorate the symptoms of cancer-induced cachexia. On the other hand, interleukin-1β (IL-1β) has been shown to impair IGF-1-dependent myoblast differentiation, resulting in the inhibition of protein synthesis and a reduction in the expression of myogenin and myosin.

AUTOPHAGY-LYSOSOMAL SYSTEM MEDIATED MUSCLE ATROPHY

The autophagy-lysosomal system (ALS) is another proteolytic system; it eliminates regions of the cytoplasm and cellular organelles by sequestering them within double-membrane vacuoles termed autophagosomes (Mizushima and Komatsu, 2011) (Figure 16.2). Subsequently autophagosomes fuse with lysosomes to form autolysosomes, followed by digestion of their contents by proteases and other lysosomal hydrolases (Mizushima and Komatsu, 2011) (Figure 16.2). The ALS is a highly conserved homeostatic mechanism used for degradation and recycling of bulk cytoplasm, long-lived proteins, and certain organelles (Mizushima and Komatsu, 2011). Autophagy is constitutively active in muscle, as shown by the accumulation of autophagosomes observed in human myopathies caused by a deficiency of lysosomal proteins, such as Pompe and Danon disease, or by pharmacological inhibition of lysosomal function, as in chloroquine myopathy. It has been reported that the ALS is critical for maintaining physiological muscle mass, as deletions in an important autophagy-related protein Atg7 result in muscle dysfunction and degeneration (Masiero *et al.*, 2009). Another autophagy-related protein, Atg5, is an E3 ligase that has been shown to be involved in removal of dysfunctional mitochondria. $Atg5^{-/-}$ T lymphocytes accumulate

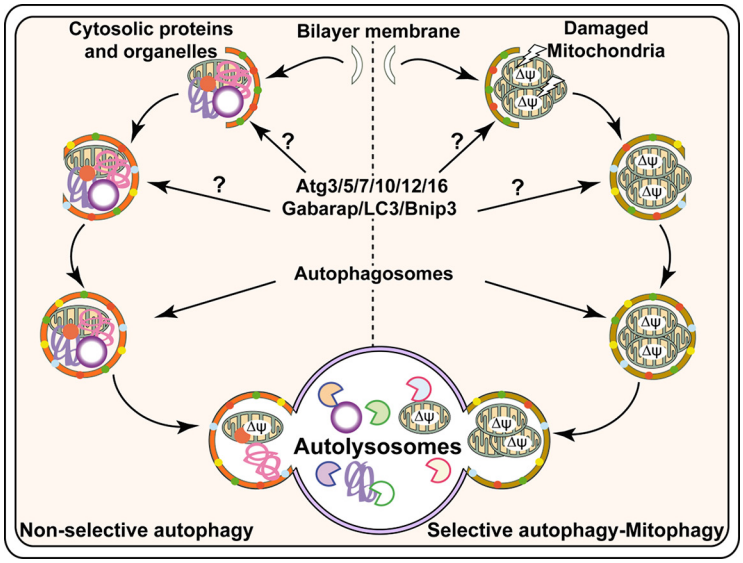

FIGURE 16.2 The two major types of autophagy – nonselective autophagy and selective autophagy (mitophagy). In the majority of circumstances, nonselective autophagy occurs when cells or tissue are deprived of nutrients. This process degrades a range of long-lived proteins, cytosolic content, and many types of organelles. Mechanistically, after the recruitment into isolation double membrane, cytosolic proteins and organelles are closed into autophagosomes with help wide variety substituent proteins (Atg3/5/7/10/12/16, Gabarapa, and LC3). Finally, the autophagosomes fuse with lysosomes for degradation by a variety of proteases and hydrolases, whereas the selective removal of mitochondria by autophagy (mitophagy) specifically removes those that are damaged or have loss of membrane potential ($\Delta\psi$) due to various reasons. Unlike in nonselective autophagy, damaged mitochondria are selectively recruited within the isolated double membrane, which closes and then fuses with lysosomes to remove the trapped mitochondria. The degradation of these components (both nonselective and selective autophagy) by hydrolases and proteases in the autolysosomes supplies building blocks for reuse and metabolism to provide ATP for cell or tissue requirements.

mitochondria that would normally be removed through autophagy of mitochondria, termed mitophagy (Sena and Chandel, 2012). Inhibition of autophagy also promotes the accumulation of nuclear abnormalities, reduces cell viability, and disrupts the function of mitochondria (Sandri, 2010).

While major metabolic organs, like the pancreas and liver, demonstrate transient activation of the ALS for several hours during fasting, muscle displays continuous production of autophagosomes for several days in response to fasting (Sandri, 2010). Activation of the ALS during muscle atrophy results in the degradation of critical autophagy components, namely LC3, a homologue of yeast Atg8 protein, as they are consumed when autophagosomes fuse with lysosomes (Figure 16.2). Hence, the continuous expression of autophagy genes is critical for ensuring that autophagy persists during periods of stress in muscle (Kroemer *et al.*, 2010). Studies conducted over the past decade have identified numerous mechanisms that modulate the activity of the ALS. The Akt pathway, for example, is a potent repressor of the ALS in muscle. Stimulation of Akt *in vivo* or in muscle cultures prevents the formation of autophagosomes and lysosome-dependent proteolysis during fasting (Zhao *et al.*, 2007).

Interestingly, Akt regulation of autophagy does not require mTOR activity, as rapamycin-induced mTOR inhibition only nominally increases protein breakdown through the autophagy-lysosomal pathway (Zhao *et al.*, 2007). Thus, current literature suggests that the activity of the ALS in muscle occurs through an mTOR-independent pathway (Masiero *et al.*, 2009). Mechanistically, when nutrients are available, mTORC1 suppresses autophagy by phosphorylating the kinase ULK1/2 and inhibits the formation of the active ULK1/2 complex (Kroemer *et al.*, 2010). In starvation conditions, mTORC1 is unable to inhibit the ULK1/2 complex, and, thus, the active ULK1/2 complex activates the ALS which allows degradation of the long-lived proteins and damaged organelles (Kroemer *et al.*, 2010).

Another negative regulator of the ALS is the transcription factor Runx1. The protective function of Runx1 was evident upon knockdown of the gene in adult muscles, whereby loss of Runx1 promoted myofibrillar disorganization and excessive autophagy in denervated muscles (Wang *et al.*, 2005). In addition, the number of autophagosomes was greater in denervated Runx1 knockout muscle when compared to wild-type muscle, further confirming that Runx1 is a potent negative regulator of autophagy in muscle (Wang *et al.*, 2005). It is currently unclear how Runx1 suppresses the activity of the autophagy-lysosomal pathway. However, recent evidence suggests a connection between Runx1 and FoxO3, another potent transcription factor involved in muscle atrophy. The FoxO transcription factors and downstream targets of IGF-1/PI3K/Akt pathway stimulate the activity of the UPS and ALS in response to muscle atrophy stimuli including starvation, denervation, and exposure to glucocorticoids such as dexamethasone (Bodine *et al.*, 2001; Sandri *et al.*, 2004). FoxO3 also induced the transcription of numerous genes involved with the ALS, such as *LC3*, *Atg7*, *Bnip3*, *Cathepsin L*, and *Gabarap1*, in response to starvation and denervation, further implicating FoxO transcription factors as master regulators of the muscle atrophy program (Romanello *et al.*, 2010; Zhao *et al.*, 2007). FoxO3 expression is sufficient and necessary for the activation of ALS-mediated protein breakdown in both animal and cell culture models. It is becoming increasingly clear that FoxO-dependent upregulation of autophagy genes is the principal mechanism by which extended periods of autophagy activity can be sustained in muscles (Masiero *et al.*, 2009). Very recently, it has been demonstrated that FoxO transcription factors also activate mitophagy through transcriptional upregulation of mitochondrial ubiquitin ligase 1 (Mul1) (Lokireddy *et al.*, 2012c).

MITOPHAGY-MEDIATED MUSCLE ATROPHY

The selective removal of malfunctioning mitochondria by ALS-mediated mechanisms is called mitophagy (Youle and Narendra, 2011) (Figure 16.2 and Figure 16.3). Recently, it was revealed that fragmentation, depolarization, or loss of mitochondria in muscle exacerbate muscle catabolism in fasted or denervated mice. Moreover, mitochondrial morphology and activity are reported to have an important role in triggering catabolic signals that contribute to muscle loss (Chen *et al.*, 2010; Romanello *et al.*, 2010).

Mitochondrial fusion and fission play a prominent role in disease-related processes such as apoptosis and mitophagy (Kroemer *et al.*, 2010) (Figure 16.3). Recently, Chen *et al.* (2010) showed that mitochondrial fusion proteins (Mfn1 and 2) are critical for mtDNA stability and mutation in skeletal muscle development. Loss of the mitofusion proteins in humans

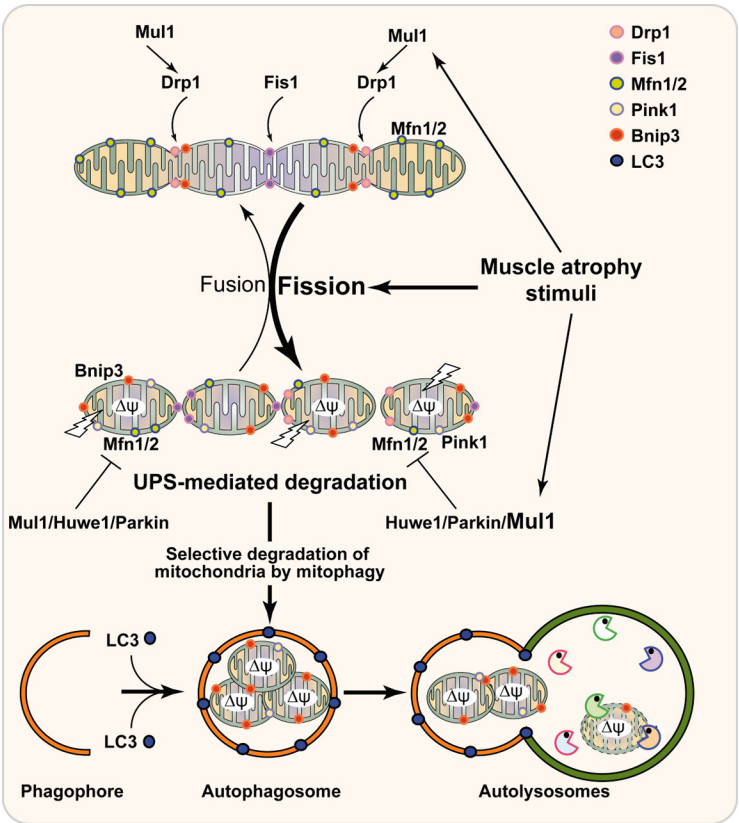

FIGURE 16.3 Molecular mechanisms behind the loss of mitochondria during muscle atrophy. Muscle atrophy stimuli, fasting, and denervation in muscle are well-documented in their ability to stimulate loss of mitochondria through selective autophagy (mitophagy). As a result of muscle atrophy stimuli, the activated FoxO transcription factors activate the critical genes involved in mitophagy processes, such as *LC3*, *Mul1*, and *Bnip3*. Mul1 overexpression promotes the fragmentation and depolarization of mitochondria and degradation of mitofusion 2 (Mfn2) proteins through UPS. Additionally, the two fission proteins, Drp1 and Fis1, are shown to be directly involved in mitochondrial fragmentation and loss of membrane potential ($\Delta\psi$) in response to muscle atrophy stimuli. As a result, there is a low ratio of fusion/fission of mitochondria during the muscle atrophy. The damaged mitochondria, which result from the loss of membrane potential and fragmented mitochondria, are then removed through mitophagy with the help of additional substitutive proteins such as PINK1/Parkin proteins. Here we propose that mitochondrial dysfunction is a potent signal that manifests skeletal muscle atrophy during the various pathophysiological conditions.

promotes the rapid and complete loss of mtDNA, resulting in absence of respiratory function (Rouzier *et al.*, 2012). The deletion or mutation of the optic atrophy protein 1 (OPA1) in mice and human results in depletion of the mtDNA nucleoids to nuclear DNA ratio (Rouzier *et al.*, 2012). Supportingly, two independent researchers, Romanello *et al.* (2010) and Lokireddy *et al.* (2012c), showed that muscle atrophy induced by food deprivation and denervation altered the morphology and activity of mitochondria and promoted loss of the organelle through mitophagy (Figure 16.3). It has also been demonstrated that activation of

FoxO transcription factors by various cachectic-like signals (treatment with dexamethasone and myostatin or exposure to serum starvation) upregulates the mitochondrial E3 ubiquitin ligase 1 (Mul1) and thus leads to increased fragmentation and disintegration of mitochondria through mitophagy (Lokireddy *et al.*, 2012c) (Figure 16.3).

Accumulation of reactive oxygen species (ROS) and oxidatively damaged proteins in cells or tissues has been linked to multiple pathologies, including neurodegenerative diseases, diabetes, cancer, and premature aging. Mitochondrial ROS were originally envisioned as an inescapable consequence of oxidative metabolism in muscle. However, there is accumulating evidence suggesting that ROS may function as signaling molecules to regulate a wide variety of processes in cells and tissues (Sena and Chandel, 2012). ROS affect signaling through a variety of cytokines, growth factors, and their downstream transcription factors such as AP-1 and NF-κB in muscle (Sena and Chandel, 2012). Recent evidence supports increased levels of ROS being detected in myoblasts and myotubes exposed to cachectic stimuli, which may be indicative of mitochondrial instability and higher membrane potential rate ($\Delta\psi$). As a result, following food deprivation, denervation, or in tumor-bearing mice, a strong decrease in the mitochondrial DNA:nuclear DNA copy number ratio was observed in muscle, indicative of mitophagy (Lokireddy *et al.*, 2012c) (Figure 16.3).

MECHANISMS UNDERLYING THE LOSS OF MITOCHONDRIA DURING MUSCLE ATROPHY

Mutations in Parkin, a ubiquitin ligase, and PINK1, a mitochondrial kinase that regulates Parkin function, are associated with the impaired clearance of defective mitochondria, which in turn results in the onset of recessive Parkinson's disease (Youle and Narendra, 2011). Studies performed in mice are consistent with the importance of the PINK1/Parkin pathway for maintaining mitochondrial integrity. Research from *Drosophila* shows that deletion of either *PINK1* or *Parkin* leads to increased oxidative stress and apparent damage to mitochondria in the flight muscle and correlates with energy deficit and increased oxidative damage of the surrounding tissue, especially in muscle fibers (Whitworth *et al.*, 2005). PINK1 senses the membrane potential of mitochondria and recruits Parkin selectively to the ones that have lost their membrane potential. Parkin subsequently ubiquitinates mitochondrial outer membrane proteins, mainly Mfn1/2 and Miro, and induces autophagic elimination of the impaired organelles through mitophagy (Narendra *et al.*, 2012) (Figure 16.3). After ubiquitination, Mfn1/2 proteins are extracted by the AAA protein p97 and then degraded by the 26S proteasome (Tanaka *et al.*, 2010). Degradation of Mfn2 has also been identified upon doxorubicin-induced stress, which triggers ubiquitination of Mfn2 by the E3 ligase Huwe1 (Leboucher *et al.*, 2012).

Recently, it has been shown that enhanced activity of the FoxO transcription factors is also associated with the disruption of mitochondrial function and organization (Lokireddy *et al.*, 2012c; Romanello *et al.*, 2010). It has also been reported that several critical mitochondrial fission proteins such as Drp1, Fis1, and Bnip3 are upregulated during fasting- and denervated-induced muscle atrophy (Lokireddy *et al.*, 2012c; Romanello *et al.*, 2010). Bnip3 is an atrogene, and it has been shown to be upregulated during muscle atrophy mediated

by FoxO3a transcription factors (Romanello *et al.*, 2010). Bnip3 has been shown to interact with Drp1 to fragment mitochondria and to regulate autophagy in skeletal muscles (Romanello *et al.*, 2010). Overexpression of fission proteins Bnip3, Drp1, and Fis1 individually or together in tibialis anterior (TA) muscles results in a strong reduction in the number of mitochondria. Current evidence suggests that mitofusins (Mfn1/2) are critical for mitochondrial integrity and furthermore, that key components of mitochondrial biogenesis, such as PGC-1α and Mfn2, were repressed in muscles isolated from both obese and nonobese diabetic patients (Liesa *et al.*, 2009).

Recently, Lokireddy *et al.* (2012c) showed that RING mitochondrial E3 ligase, Mul1 (Mitochondrial E3 ubiquitin ligase 1) induces the fragmentation, depolarization, and clearance of mitochondria through the autophagy-lysosome pathway during *in vivo* muscle atrophy (Lokireddy *et al.*, 2012c). Like most other mitochondrial proteins, Mul1 is encoded in the nuclear genome and localized to the outer membrane of mitochondria. Mul1 possesses two transmembrane domains and a C-terminal RING finger domain, and functions to regulate the fusion and fission of mitochondria (Braschi *et al.*, 2009; Zhang *et al.*, 2008). Mul1 is expressed ubiquitously, but most abundantly in cardiac and skeletal muscle (Zhang *et al.*, 2008). The RING finger domain of Mul1 ubiquitinates and targets the Mfn2 for UPS-mediated degradation in atrophying muscle (Lokireddy *et al.*, 2012c). Mul1 overexpression during muscle atrophy is regulated by FoxO3 transcription factors in muscle (Lokireddy *et al.*, 2012c). Ectopic overexpression of Mul1 promotes fragmentation, depolarization, and removal of mitochondria through mitophagy. According to previously published reports, Mul1 has been shown to stabilize Drp1, a mitochondrial fission protein, resulting in mitochondrial fragmentation (Braschi *et al.*, 2009). Since it is expressed in all tissues, it would be interesting to learn how Mul1 regulates the mitochondrial function in all other tissues using the tissue-specific or conditional knockout mouse models.

CONCLUSION AND FUTURE DIRECTIONS

TGF-β family growth factors, activin A and myostatin, are regarded as attractive drug targets, since therapeutics that stimulates skeletal muscle growth may ameliorate the effect of muscle atrophy conditions such as muscular dystrophy, sarcopenia, cancer cachexia, and various muscle atrophies. In addition to negative function of activin A and myostatin, fasting- and denervation-induced muscle atrophy primarily occurs due to the elevated activity of the ubiquitin-proteasome and autophagy-lysosomal systems. More recent evidence suggests that mitochondrial instability also plays a prominent role in mediating denervation- and fasting-induced muscle atrophy. In this chapter, we have discussed how autophagy and mitophagy both play critical roles in the removal of malfunctioning mitochondria during starvation- and denervation-induced muscle atrophy. Given that mitochondrial dysfunction is also a characteristic feature of metabolic diseases like diabetes, diet-induced obesity, and sarcopenia, one can speculate that mitophagy may play a major role in the onset of such pathologies. In the future, it would be interesting to investigate the role of mitophagy during various metabolic disorders in muscle using tissue-specific conditional knockout or transgenic mice for genes involved in mitophagy mechanisms.

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CHAPTER

17

Role of Impaired Mitochondrial Autophagy in Cardiac Aging

Mechanisms and Therapeutic Implications

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OUTLINE

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Abstract

Advanced age *per se* is a major risk factor for cardiovascular disease (CVD). Apart from the chronic exposure to known cardiovascular risk factors, intrinsic aging of the cardiovascular system amplifies the vulnerability to various stressors, ultimately favoring the development of CVD in late life. The process of cardiac aging involves derangements in multiple cellular pathways. Among these, impairments in mitochondrial function arising from failure of mitochondrial quality control are considered to be a major contributing factor to heart senescence. Besides being less bioenergetically efficient, damaged mitochondria produce larger amounts of reactive oxygen species and are more prone to induce apoptosis. This results in oxidative damage to cardiomyocyte components and dismissal of postmitotic and virtually irreplaceable cardiac cells. The chapter summarizes the current knowledge about prominent mechanisms and consequences of mitochondrial decay and failure of mitochondrial quality control in the context of cardiac aging. The therapeutic potential of targeting specific mitochondrial pathways is also discussed.

INTRODUCTION

Advanced age is a major risk factor for virtually all chronic degenerative diseases, including cardiovascular disease (CVD). This is reflected by the fact that over 80% of cases of coronary artery disease and more than 75% of those of congestive heart failure (CHF) are observed in elderly patients (Lloyd-Jones *et al.*, 2009).

Undoubtedly, the long-term exposure to cardiovascular risk factors plays a major role in the etiopathogenesis of CVD in advanced age; however, intrinsic cardiac aging, defined as the occurrence of anatomical and functional changes in the heart over the lifetime, amplifies the risk for CVD in advanced age (Dutta *et al.*, 2012). Relevant age-related changes include a 35% loss of cardiomyocytes and a 20% decrease of sinoatrial nodal pacemaker cells due to apoptosis, necrosis, and possibly autophagy, accompanied by reactive hypertrophy of the remaining cardiomyocytes (Olivetti *et al.*, 1991). The loss of cardiac cells is also paralleled by a decline in regenerative activity from 1% per year at age 20 years to 0.4% at the age of 75 (Bergmann *et al.*, 2009).

The mechanisms involved in cardiovascular senescence are not completely understood. Nevertheless, alterations in mitochondrial function are widely considered to be a major contributing factor (Marzetti *et al.*, 2009). Dysfunctional mitochondria not only are less bioenergetically efficient, but also generate increased amounts of reactive oxygen species (ROS), interfere with cellular quality control (QC) mechanisms, and are more prone to trigger apoptosis (Dutta *et al.*, 2012). Due to the detrimental consequences of mitochondrial dysfunction, the removal of damaged mitochondria and their replacement with newly formed organelles are crucial for the maintenance of cellular homeostasis. This is especially true for postmitotic adult cardiomyocytes, in which energy provision is highly dependent on mitochondrial oxidative phosphorylation, and mitochondria-derived oxidative damage cannot be effectively diluted through cell proliferation. In this context, recent evidence indicates that the efficiency of mitochondrial turnover declines in the aging heart, which may have important consequences for cardiovascular senescence and CVD (Dutta *et al.*, 2012). In this chapter, we discuss relevant mechanisms whereby cardiac mitochondria become dysfunctional in advanced age and how defective mitochondrial autophagy may contribute to cardiovascular aging.

MECHANISMS AND CONSEQUENCES OF MITOCHONDRIAL DYSFUNCTION IN THE AGING HEART

Mitochondria as a Source of Oxidants

The heart is highly energy dependent and about 90% of the energy consumed is supplied by mitochondrial oxidative phosphorylation. This implies that the proper functioning of myocardial mitochondria is crucial for optimal cardiac function. Free radicals are constantly generated during mitochondrial respiration (Figure 17.1). However, if mitochondrial antioxidant defenses are fully functioning and electron leakage from the electron transport chain (ETC) occurs within the physiologic range, oxidative damage is almost completely prevented. In such circumstances, the small amounts of ROS generated function as second-messenger molecules that modulate the expression of several genes involved in metabolic regulation and stress resistance (mitochondrial hormesis or mitohormesis) (Handy and Loscalzo, 2012). On the other hand, excessive ROS generation and/or defective oxidant scavenging are thought to contribute to the aging process as well as to the pathogenesis of several chronic degenerative diseases, including CVD (Judge and Leeuwenburgh, 2007). The following subsections illustrate the mechanisms responsible for abnormal mitochondrial oxidant generation during cardiac aging and highlight the impact of oxidative stress on heart physiology.

Mechanisms of Mitochondrial Dysfunction in the Aging Heart

Contrary to normally functioning mitochondria, from which oxidant emission is negligible, damaged cardiac mitochondria can release large amount of ROS (up to 10-fold more relative to intact organelles) (Grivennikova *et al.*, 2010). Furthermore, in the presence of

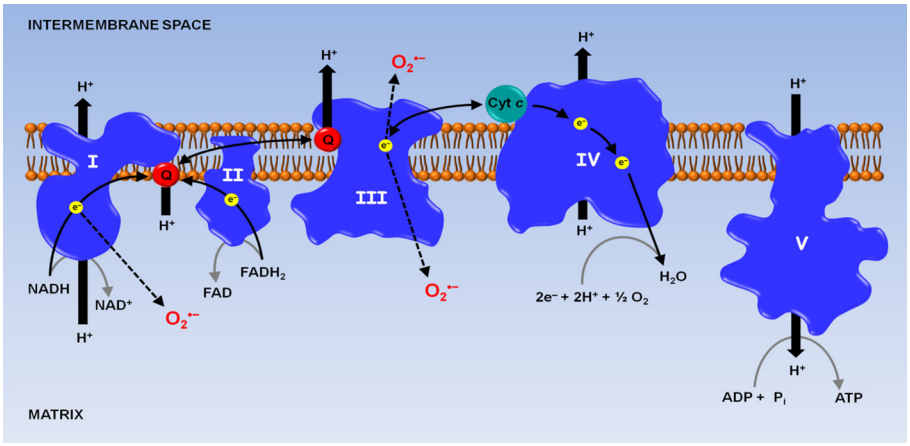

FIGURE 17.1 Generation of reactive oxygen species by the electron transport chain. The production of superoxide anion ($O_2^{\cdot-}$) occurs mainly at complexes I and III. $O_2^{\cdot-}$ generated by complex I diffuses into the matrix. At complex III, $O_2^{\cdot-}$ can be released into both the matrix and the intermembrane space.

non-protein-bound redox cycling metals (e.g., iron and copper), mitochondria-derived hydrogen peroxide (H_2O_2) can be converted into the highly reactive hydroxyl radical ($^{\bullet}OH$), through Fenton's and Haber-Weiss's reactions. It is worth mentioning that mitochondrial iron content increases with aging in rodent postmitotic tissues, including the heart, which may amplify the extent of oxidative damage by enhancing the generation of highly reactive species, such as $^{\bullet}OH$ (Xu *et al.*, 2010). In such circumstances, the mitochondrion is exposed to a high burden of ROS, which results in the primary damage to its own constituents. Indeed, mitochondria are highly susceptible to the oxidative stress they generate. This is mostly due to the close proximity of mitochondrial DNA (mtDNA) to the site of ROS generation, the lack of protective histones, and a somewhat less efficient DNA repair system compared with nuclear DNA (Yakes and Van Houten, 1997).

ROS-inflicted mtDNA mutations can lead to the synthesis of defective ETC components, resulting in bioenergetic efficiency and further ROS generation (Miquel *et al.*, 1980). This vicious cycle is believed to play a central role in the aging process as well as in the pathogenesis of age-related degenerative diseases, including CVD (Miquel *et al.*, 1980). Interestingly, recent experimental evidence has linked the mitochondrial free radical and the telomere shortening theories of aging (Sahin *et al.*, 2011). Mice with impaired telomere maintenance caused by targeted deletion of telomerase reverse transcriptase ($Tert^{-/-}$) develop severe telomere dysfunction when backcrossed for four or more generations (G4). This is causative for pathologies not only in highly proliferative tissues, but also in postmitotic organs, such as the heart. Indeed, G4 $Tert^{-/-}$ mice develop dilated cardiomyopathy during aging, with left ventricular wall thinning and reduced contractile performance. Cardiac mitochondria from G4 $Tert^{-/-}$ mice exhibit reduced mtDNA abundance, impaired respiration, and increased ROS generation. These abnormalities are linked to p53-mediated repression of peroxisome proliferator-activated receptor-γ coactivator-1α and 1β (PGC-1α and PGC-1β) and their downstream targets nuclear respiratory factor-1 (NRF-1) and mitochondrial transcription factor A (TFAM). The age-related telomerase dysfunction might therefore act as the *primum movens* leading to mitochondrial decay, which in turn would result in decreased bioenergetic efficiency and increased ROS production through sustained p53 activation and further repression of PGC-1 signaling.

Another relevant consequence of a dysfunctional telomere-p53-PGC axis is the impairment in mitochondrial biogenesis. Mitochondriogenesis is coordinated by a set of coactivators and transcription factors. In this process, PGC-1α and PGC-1β function as master regulators by promoting the expression of transcription factors, including NRF-1 and TFAM, which are then responsible for the synthesis of nuclear-encoded proteins and the transcription and replication of the mitochondrial genome, respectively. Hence, the disruption of the PGC-1 signaling cascade may contribute to the reduced cardiac mitochondrial biogenesis observed during aging (Qi *et al.*, 2011).

The involvement of the mitochondrial free radical vicious cycle in cardiac aging has received further support from findings in mice that express a proofreading-deficient mtDNA polymerase γ (PolG) (Kujoth *et al.* 2005). These rodents accumulate a high load of mtDNA mutations and deletions in the heart, in conjunction with the early onset of several age-associated changes, such as cardiac enlargement, fibrosis, and impairment of systolic and diastolic function. At the cellular level, mtDNA-mutator mice exhibit accumulation of swollen and irregularly shaped mitochondria with impaired ETC activity and increased

levels of protein carbonyls, a marker for protein oxidation (Dai *et al*. 2010). PolG mice die prematurely from dilated cardiomyopathy, an outcome observed also in mice expressing a cardiac-specific proofreading-deficient PolG (Zhang *et al*., 2003).

A further proof of principle for the role of mitochondria-derived oxidants in cardiac aging has been provided by the characterization of mice with overexpression of the antioxidant enzyme catalase targeted to the mitochondrial matrix (mCAT) (Dai *et al*. 2009). At the cellular level, these animals are characterized by decreased rates of H_2O_2 production and reduced mitochondrial oxidative damage and mtDNA deletions. This translates into attenuation of age-related cardiac enlargement, delayed development of heart pathology, and increase in mean and maximum lifespan. Remarkably, the PolG heart phenotype, the cardiac mtDNA mutation load, and the extent of mitochondrial protein oxidation are partially rescued by mCAT overexpression (Dai *et al*., 2010).

As a whole, studies in rodent models have made a strong argument in favor of the mitochondrial vicious cycle as a contributing factor to cardiac senescence. Nonetheless, definite evidence of the involvement of mitochondrial decay in the aging process requires the reciprocal experiment – that is, the generation of rodents engineered to suffer a reduced rate of mtDNA mutations during aging. If these animals showed normal mitochondrial function and heart performance in late life, the contribution of mitochondrial damage to cardiac senescence would be established conclusively.

Consequences of Mitochondrial Dysfunction on Heart Physiology

Enlarged mitochondria, characterized by ultrastructural abnormalities and increased ROS generation, have been observed in the myocardium of old rodents in conjunction with elevated levels of oxidative damage to mitochondrial proteins, lipids, and nucleic acids (Sachs *et al*., 1977; Sohal *et al*., 1994). With regard to mtDNA, the frequency of point mutations and deletions is approximately 3-fold higher in old mouse hearts relative to young adult controls (Dai and Rabinovitch, 2009). An age-dependent increase in cardiac mtDNA damage has also been shown in humans. The frequency of the common 4977-bp mtDNA deletion, a typical consequence of oxidative damage, increases with age in the human heart and is estimated to be 5- to 15-fold higher in persons over 40 years of age as compared to younger controls (Mohamed *et al*., 2006). The bioenergetic consequences of the accumulation of 4977-bp deletions arise when the proportion of deleted mtDNA exceeds 50–55% of total mtDNA, and include declines in mitochondrial membrane potential and ATP synthesis and increases in ROS generation. Damaged mitochondria are also more prone to induce apoptosis, with subsequent loss of cardiac cells (Kujoth *et al*. 2005). Indeed, cardiomyocyte removal through apoptosis increases with advancing age, which, combined with insufficient stem cell replenishment, may contribute to impairing the contractility of the aging heart (Marzetti *et al*., 2009) (Figure 17.2).

It is important to note that the interpretation of age-related changes in cardiac mitochondrial function is complicated by the fact that two distinct populations of mitochondria exist in the myocardium: subsarcolemmal mitochondria (SSM), located beneath the plasma membrane, and interfibrillar mitochondria (IFM), arranged between the myofibrils.

Cardiac IFM isolated from old rats exhibit reduced rates of oxidative phosphorylation relative to young adult controls, with no differences for the SSM subpopulation between

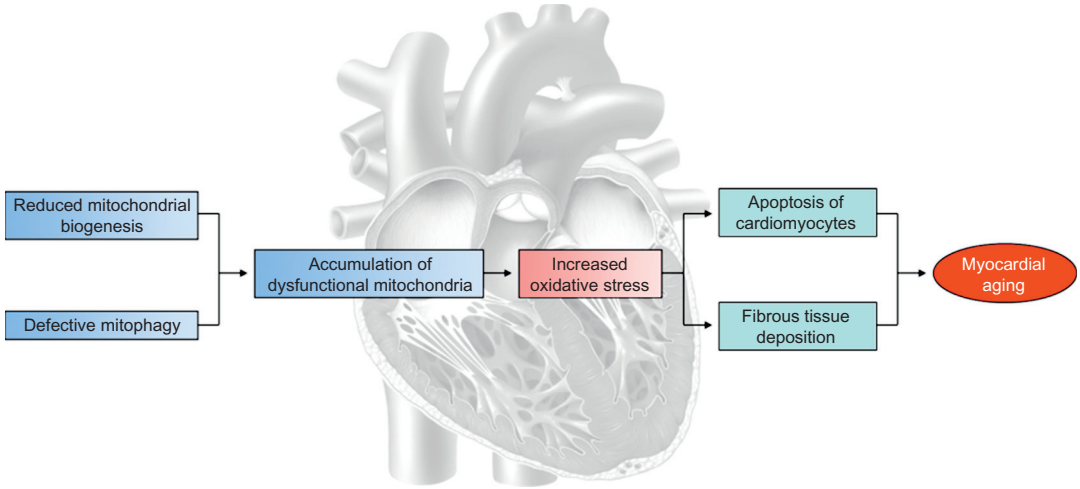

FIGURE 17.2 Proposed scenario of mitochondrial quality control failure, organellar dysfunction and triggering of mitochondrion-mediated apoptosis during cardiac aging. Reduced mitochondrial biogenesis and defective autophagy lead to the accumulation of dysfunctional, ROS-leaking mitochondria. Excessive oxidant production eventually triggers apoptosis of cardiomyocytes and promotes the deposition of fibrous tissue within the myocardium.

age groups (Fannin *et al.*, 1999). In contrast, H_2O_2 generation increases with age in SSM, but not IFM (Judge *et al.*, 2005). These results are in contrast to previous findings by others who reported an age-dependent increase in oxidant production by IFM, but not SSM isolated from rat hearts (Suh *et al.*, 2003). This opposite evidence could originate from methodological differences between the two studies with regard to the assessment of mitochondrial oxidant production.

IFM from aged rat hearts show an increased susceptibility toward opening of the mitochondrial transition pore (mPTP), an event upstream of apoptosis and necrosis (Hofer *et al.*, 2009). However, it is currently unclear whether the two mitochondrial populations are involved differently in the removal of old cardiomyocytes.

The evaluation of the available data on age-related biochemical differences between IFM and SSM is further complicated by the fact that most isolation procedures yield either SSM alone or a mixed population of SSM and IFM. This may help explain the lack of consistency regarding age-related changes in bioenergetics and ROS generation among the various studies. Finally, cardiomyocytes with extremely dysfunctional mitochondria are likely eliminated via apoptosis and/or necrosis, so that only relatively healthy mitochondria are obtained upon isolation.

The available evidence supports the therapeutic potential of improving mitochondrial redox homeostasis to prevent or delay cardiac aging. Nevertheless, supplementation with antioxidants has led to little or no cardioprotection in humans. A great deal of attention has therefore been diverted toward the optimization of mitochondrial QC to repair and/or remove damaged mitochondria, as discussed in the next paragraph.

CONTRIBUTION OF IMPAIRED MITOCHONDRIAL QUALITY CONTROL TO CARDIAC AGING

The maintenance of a functional mitochondrial pool within cardiomyocytes relies on the efficiency of QC processes, which are responsible for repairing or eliminating dysfunctional organelles. For instance, fusion and fission of mitochondria prevent the local accumulation of dysfunctional organelles and segregate from the vital mitochondrial pool those that are irreversibly damaged or unnecessary (Twig *et al*., 2008). A specialized form of autophagy, mitophagy (*vide infra*), degrades mitochondria segregated by fission and is therefore placed at the end of the mitochondrial QC axis (Twig *et al*., 2008). The mechanisms whereby altered mitochondrial dynamics and autophagy are believed to contribute to cardiovascular aging and CVD are described in the following subsections.

Role of Altered Mitochondrial Dynamics in Cardiac Aging

Once considered to be static, isolated organelles, mitochondria are now viewed as highly mobile entities that form dynamic networks within cells. The dynamic behavior of these networks influences the bioenergetic status of the cell and allows it to cope with changing stresses and metabolic demands.

The morphology and function of mitochondrial networks are regulated by coordinated fusion and fission events, for a detailed description of which the reader is referred to specialized reviews (for instance, Youle and van der Bliek, 2012). Fusion and fission serve crucial roles in the redistribution of metabolites and proteins, maintenance of mtDNA integrity, regulation of metabolic processes, and modulation of QC and cell death pathways. For instance, the functionality of damaged mitochondria can be complemented, or even restored, by their fusion with neighboring intact mitochondria (Twig *et al*., 2008). Conversely, irreversibly damaged or unnecessary mitochondria are segregated from the network through fission and eventually eliminated via mitophagy (Twig *et al*., 2008). Given these vital functions, it is not surprising that alterations in mitochondrial dynamics are invoked as a contributing factor to cardiovascular senescence and CVD (Marzetti *et al*., 2013).

In adult cardiomyocytes, mitochondria, at least IFM, are organized into a regular, crystal-like lattice arrangement. In spite of this peculiar arrangement, fusion and fission are involved in the regulation of energy homeostasis in the adult heart, especially under stress conditions (Piquereau *et al*., 2012). Dysregulation of mitochondrial dynamics may play a role in the pathogenesis of several conditions, such as cardiomyocyte hypertrophy, myocardial ischemia/reperfusion (I/R) injury, and CHF (Ong *et al*., 2012). For instance, excessive mitochondrial fission (and/or decreased fusion) may be detrimental in I/R injury, diabetes, and heart failure (Ong *et al*., 2012). In contrast, inhibition of fission is cardioprotective during I/R (Ong *et al*., 2010). These findings may indicate that, under conditions of enhanced oxidative stress, inhibition of fission and possibly upregulation of fusion could occur to withstand the increasing load of mtDNA mutations. It is tempting to speculate that a similar adaptation may be put forward during aging; however, this hypothesis has yet to be explored.

In conclusion, the available evidence, although very preliminary, suggests that manipulation of mitochondrial dynamics may represent a novel pharmacological target against

cardiac aging and CVD. However, a deeper understanding of the function of mitochondrial fission and fusion in health and disease is required before these pathways can be harnessed to achieve cardioprotection.

Contribution of Altered Mitochondrial Autophagy to Heart Senescence

Autophagy is a self-digesting process through which cells dispose of their own components, recycling amino acids and other building blocks that eventually can be reused. Macroautophagy (herein referred to as autophagy) is the best-characterized autophagic pathway in mammalian cells, and consists of a genetically programmed, evolutionarily conserved catabolic process that degrades damaged or unnecessary cellular proteins and organelles through their sequestration into a double-membrane structure known as the autophagosome. The latter fuses with lysosomes to form an autolysosome wherein the cargo is degraded.

Until recently, autophagy was considered a random, bulk degradation process. Yet, it has become clear that autophagy can proceed either unselectively or selectively. In response to starvation, nonselective autophagy is activated which serves to provide cells with nutrients necessary for survival. Selective autophagy functions to specifically remove protein aggregates or damaged or superfluous organelles, and is operative independent of nutrient conditions. The term *mitophagy* identifies a specialized pathway of selective autophagy that is specifically targeted toward the degradation of mitochondria. Mitophagy regulates mitochondrial number to match metabolic demands and also serves as a QC mechanism to remove damaged mitochondria (Figure 17.3). Mitophagy is preceded by mitochondrial fission, which segregates mitochondria from the network for subsequent disposal. For a detailed description of the molecular machinery of autophagy and mitophagy and the regulation of these pathways, the reader is referred to specialized reviews (for instance, Wang and Klionsky, 2011; Yang and Klionsky, 2010).

Since damaged mitochondria accumulate during aging, a concomitant increase in mitophagic flux and replenishment with newly formed organelles could be expected to act protectively. However, the efficiency of autophagy declines in late life, which may have important implications for highly energy-dependent tissues, such as the heart (Dutta *et al.*, 2012) (Figure 17.2). Over time, this autophagic failure may result in the accumulation within cardiomyocytes of dysfunctional mitochondria that are bioenergetically inefficient and prone to ROS leakage. Indeed, ultrastructural and biochemical analyses of myocardium from old rodents have revealed the presence of enlarged mitochondria, characterized by swelling, loss of cristae, matrix derangement, reduced ATP production, and increased ROS generation (Sachs *et al.*, 1977). What is more, giant mitochondria are thought to progressively displace functional organelles over the course of aging, due to a replicative advantage of damaged mitochondria with a partially deleted genome (Dirks *et al.*, 2006). Enlarged mitochondria may also benefit from a survival advantage, being less likely to be engulfed and degraded due to their size (Dirks *et al.*, 2006).

In addition to the accumulation of damaged mitochondria, the myocardium of old rodents is characterized by the buildup of a nondegradable pigment called lipofuscin or age pigment (Dutta *et al.*, 2012). Lipofuscigenesis results from peroxide-induced Fenton reactions triggered by intralysosomal materials. ROS-inflicted modifications to proteins

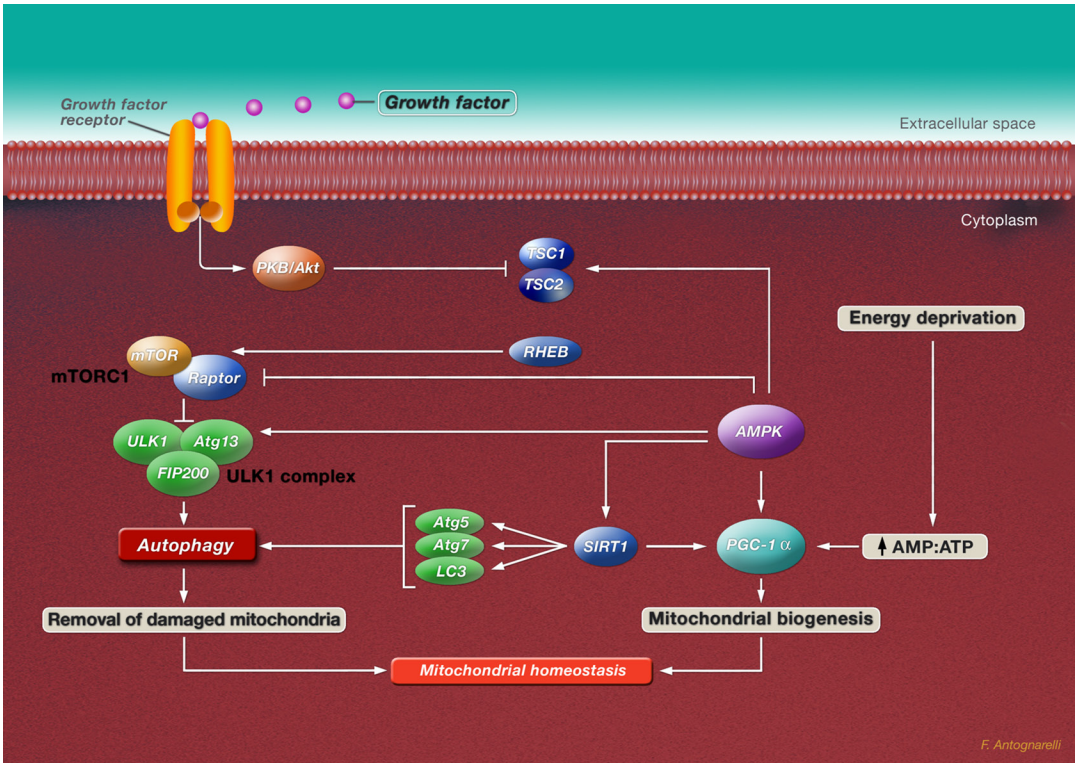

FIGURE 17.3 Overview of the molecular regulation of mitochondrial autophagy and biogenesis. In the presence of growth factors, the protein kinase B (PKB/Akt) pathway is activated and blocks the tuberous sclerosis complex 1/2 (TSC1/TSC2), thereby relieving its inhibitory effect on Ras-homologue enriched in brain (RHEB). RHEB activates the mammalian target of rapamycin complex 1 (mTORC1), which inhibits autophagy by blocking the ULK1-Atg13-FIP100 complex. Under energy depletion, the AMP-to-ATP ratio increases and the AMP-activated protein kinase (AMPK) becomes activated, which stimulates autophagy. AMPK can activate TSC1/TSC2, thereby relieving the mTORC1-mediated inhibition of autophagy. In addition, AMPK can phosphorylate ULK1 at specific serine residues, thereby activating autophagy. AMPK can also inhibit the mTORC1 complex by phosphorylating the mTORC1 containing rapamycin-associated TOR protein (Raptor), the binding partner required for mTORC1 activity. AMPK also induces mitochondrial biogenesis by activating peroxisome proliferator-activated receptor γ coactivator 1α(PGC-1α) either directly or via sirtuin-1 (SIRT1). SIRT1 can also deacetylate and activate several autophagy-related (Atg) proteins, including Atg5, Atg7, and LC3. The coordination between mitochondrial autophagy and biogenesis is essential for the maintenance of mitochondrial homeostasis. *Artwork by Francesco Antognarelli.*

and lipids cause crosslinking inside lysosomes/autolysosomes, which eventually generates lipofuscin. Peroxides involved in these Fenton reactions can diffuse into lysosomes from cytosolic damaged mitochondria or can originate from autophagocytosed, yet undegraded, mitochondria. Lipofuscin-loaded lysosomes consume a large part of newly produced lysosomal hydrolases that, however, cannot digest lipofuscin. At the same time, a smaller amount of lysosomal enzymes remains available for autophagic pathways, including mitophagy (Brunk and Terman, 2002). Hence, autophagic failure and the accumulation

of damaged mitochondria perpetuate each other, resulting in further oxidative stress and lipofuscinogenesis and, eventually, collapse of the cell catabolic machinery (garbage catastrophe theory of aging, also known as the mitochondrial-lysosomal axis theory of aging) (Brunk and Terman, 2002).

Although recent evidence supports the involvement of impaired mitochondrial autophagy in cardiac aging, several research questions remain unanswered. First, the optimal level of autophagy activation to achieve cardioprotection is currently unclear. While it stands reasonable to hypothesize that upregulation of cardiomyocyte autophagy might be beneficial during aging, studies have shown that excessive activation of this pathway may be maladaptive and contribute to the progression of heart failure. More importantly, whether the optimization of autophagy preserves mitochondrial function and delays cardiac aging in humans is presently unknown. By answering these and other critical questions it will likely allow clinicians to delay cardiac aging and achieve cardioprotection through the fine tuning of autophagy, avoiding the detrimental consequences of its defective or excessive activation.

MITOCHONDRIAL DYSFUNCTION AND QUALITY CONTROL: NOVEL PHARMACOLOGICAL TARGETS AGAINST CARDIAC AGING AND CARDIOVASCULAR DISEASE

The central role attributed to mitochondria-driven oxidative damage in cardiac aging and CVD would suggest that the administration of antioxidants might mitigate the burden of CVD in late life. Yet, most clinical trials that have tested the effects of systemic, nonselective supplementation with antioxidants to attenuate the progression of CVD failed to achieve any positive cardiovascular outcome. In contrast, the selective delivery of molecules with antioxidant properties to mitochondria has shown efficacy in models of cardiovascular stress (Subramanian *et al.*, 2010). Examples of such molecules include Szeto-Schiller (SS) synthetic antioxidant peptides, 10-(6′-ubiquinonyl)-decyltriphenylphosphonium (MitoQ), and Euk-8 (a SOD/catalase mimetic and antioxidant) (Subramanian *et al.*, 2010). Several clinical studies are currently ongoing to test the efficacy of mitochondria-targeted antioxidants in various CVD conditions.

The development of interventions targeting mitochondrial QC is another promising approach to counter age-dependent cardiovascular degeneration. In this context, calorie restriction (CR), resveratrol administration, and sirtuin(s) pathway(s) activation could be especially relevant to delay cardiac aging and manage age-related CVD (Dutta *et al.*, 2012). CR, defined as a reduction in food intake without malnutrition, confers cardiovascular protection during aging and in pathological conditions associated with accelerated cardiac aging (Marzetti *et al.*, 2009). CR conveys beneficial effects on the cardiovascular system through different mechanisms, including attenuation of mitochondrial ROS production and optimization of mitochondrial autophagy (Marzetti *et al.*, 2009). It is also proposed that CR improves mitochondrial biogenesis, but this point remains controversial. Despite the host of health benefits brought about by CR, it is likely that most people will not be able to sustain drastic food restrictions for the long term. Moreover, chronic CR has been associated with several adverse events. Thus, considerable effort has been directed toward the discovery of drugs that could mimic the effects of CR without requiring dietary restriction. Among CR

mimetics, the polyphenol resveratrol (3,5,4′-trihydroxystilbene) has been the most extensively investigated. This compound is believed to be responsible for the cardioprotective effects of red wine. Studies in rodents have shown that resveratrol inhibits cardiomyocyte apoptosis, protects the myocardium against I/R injury, prevents left ventricular hypertrophy, and reduces inflammation (Marzetti *et al.*, 2009). Resveratrol has also been shown to recapitulate the cardioprotective adaptations induced by CR, including the optimization of autophagy and mitochondrial turnover and the attenuation of mitochondrial oxidative stress (Marzetti *et al.*, 2009).

Another popular CR mimetic, the antidiabetic drug metformin, has shown to ameliorate myocardial ischemia, infarction severity, diabetic cardiomyopathy, and heart failure in laboratory rodents (El Messaoudi *et al.*, 2011). Furthermore, metformin administration improves age-related contractile defects in murine cardiomyocytes. Similarly to resveratrol, cardioprotection by metformin appears to occur via optimization of mitochondrial function, restoration of autophagic efficiency, and decreases in oxidative stress.

In conclusion, preclinical studies indicate that interventions aimed at reducing mitochondrial-specific oxidative burden, improving mitochondrial function, and optimizing mitochondrial turnover represent a promising strategy to manage cardiovascular deterioration with age. Further investigations are warranted to determine whether these interventions preserve mitochondrial function and promote cardiovascular health in older persons.

CONCLUSION

Advancing age *per se* is a major risk factor for CVD. Mitochondrial decay is believed to play a critical role in the pathogenesis of intrinsic cardiovascular aging. Given the postmitotic nature of cardiomyocytes, the optimal regulation of mitochondrial turnover is critical for the maintenance of cell viability. Mitochondrial autophagy and biogenesis serve this essential homeostatic function. However, the efficiency of these processes declines with advancing age, which may have relevant implications for cardiovascular aging. Indeed, accumulating evidence suggests that defective mitochondrial QC can contribute to abnormal oxidant generation, disruption of bioenergetics, and accrual of intracellular waste material in the aging heart. At the same time, mitochondrial autophagy has emerged as a promising pharmacological target against cardiac aging and age-related CVD.

It should, however, be considered that autophagy (and probably mitophagy) exhibits a dual behavior. While basal levels of autophagy are fundamental for maintaining cellular homeostasis and protecting against the accumulation of dysfunctional mitochondria, an abnormal upregulation of autophagy can lead to excessive removal of mitochondria, loss of cardiomyocytes, and development of CVD.

A growing number of studies have been exploring the possibility of delaying cardiac aging and treating CVD by targeting specific mitochondrial pathways. For instance, mitochondria-targeted antioxidants have been shown to confer cardioprotection in various animal models. In addition, pharmacological or nutritional interventions (e.g., CR and CR mimetics like resveratrol and metformin) that upregulate intrinsic antioxidant systems in mitochondria and optimize mitochondrial QC appear to offer therapeutic advantage in age-related cardiac degeneration and CVD.

In summary, an extraordinary research effort is required to untangle the complexity of the network of multileveled interrelated pathways that regulate mitochondrial homeostasis. This knowledge will likely provide clinicians with novel and highly effective therapeutics to delay cardiovascular aging and manage CVD.

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