AUTOPHAGY

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

VOLUME 8

Edited by

M. A. Hayat
Distinguished Professor
Department of Biological Sciences
Kean University
Union, New Jersey

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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

Autophagy and Cancer

When speaking of cancer, autophagy's good
By culling mitochondria and clearing deadwood
Autophagy limits the radical chain
That breaks DNA and mutates a gene
That makes a cell double, so careless and mean
In order for cells to malignant transform
They lose mitochondria except for a few
Using glycolysis as the source of their fuel
How they achieve mitochondrial decimation
Is nothing more than autophagic elimination.

Then one cell is many, an ominous mass
Demanding more glucose, hungry and crass,
Directing formation of artery and vein
'Til capsular fibers give way under strain
Then cancer cells spread so far and so wide
They demand blood vessels the body provide
But until those are patent the tumor cells strive
To rely on autophagy to neatly survive
The hurdles required for metastasis
Until blood flow's established for cancerous bliss.

Blocking autophagy sends them over the brink
And how chloroquine works, we think
But tumors are slowed by statin's effects
Which induce autophagy and tumor cell death
Autophagy's good, autophagy's bad
The confusion's enough to drive us all mad
So study we must, and learn ever more
'Til enlightenment finally opens the door
Oncologists must heed the tumor's agenda
And decide whether autophagy is a foe or friend.

Roberta A. Gottlieb

Some Thoughts on Autophagy and Immunity

A bacterium squirmed into a cell
Thinking "This home will serve me well"
The cell objected quite forcefully
Encasing the bug in LC3
Saying "I'm not your home,
You're imprisoned in my autophagosome!"
The bug merely shrugged and secreted a factor
Poking holes in the shell, releasing the actor
Who by now had multiplied so many times
They were all ready to commit more devious crimes.

Autophagy's a way to lock those critters away
But bugs evolve too, and have learned what to do
To turn host defense to their convenience.
So mark my words and mark them well
If you want to be a clever cell
Turn autophagy up to kill pathogen C, D, or E
But keep it turned down for bugs A, B, and D.
How to do that?
Eating no meat and eating no fat
Will turn up the autophagy thermostat.
But sugar and fat and protein too
Will slow it down as good as glue.
Remember a rich diet keeps autophagy quiet
Skip brunch and sup to turn autophagy up.

Trouble comes as the number one,
If it's interleukin-1….Beta, that is.
Relief comes as the number three
LC3….B, that is.
Letters and numbers, numbers and letters
Stop getting dumber and learn from your betters.
Autophagy works to prevent calamity
By turning down inflammity.

Autophagy's a way to share information
From macrophages by antigen presentation
To lymphocytes of each denomination
When properly goaded, MHC-IIs are loaded
With tasty bites of foreign peptides.
Endosome to lysosome
Bits of the stranger are made known
To help program immunity,
Thanks be to you, autophagy!

Roberta A. Gottlieb

Autophagy: Friend or Foe?

Be careful when hugging Atg5
It can help you get dead or be live.
When Atg12's covalently bound
Autophagy's up and death can't be found.
But if protease scissors free the BH3
Fragment of Atg5, soon you will see
Death and destruction, known as
Programmed cell death or apoptosis.

Beclin 1 is capricious too,
Hitching itself to Bcl-2.
In this way it deflects
Bcl-2's survival effects.
But helped by VPS34
It forms a phagophore:
Autophagy goes well
And rescues the cell.

Roberta A. Gottlieb

Autophagy: If and When

Like a foreign body embedded in scar
Membrane shrouds the mitochondrion
Lest it activate the inflammasome
And trigger release of IL-1.

Of course we need an antidote
To avoid the damage from mitos gone bad
Enter the savior—mitophagy,
Rescuing the cell like Sir Galahad.

Another case where autophagy's good
Is in the aging, sludgy brain
Where it serves to clear proteinaceous crud:
Autophagy to the rescue, once again.

The bleakest core of a malignant mass
Like a hypoxic inner circle of Hell
Is where autophagy plays a darker role
To aid in the survival of cancer cells.

Do we want to trigger autophagy?
We need to know how, where, and when.
Read this book of cellular wizardry
And then you'll know: It all depends!

Roberta A. Gottlieb

What Happened When Autophagy Didn't

A mito decayed and leaked DNA
Plus cytochrome *c* and 8-oxo-dG
The inflammasome blew
And the apoptosome too
And the cell had a very bad day.

Roberta A. Gottlieb

Sugar Isn't Always Sweet

Sugar isn't always sweet,
When your heart is worn and skips a beat
Membranes keep it in or out
And insulin gives a special route

Glut transporters form a pore
Bringing sugar in the open door
Too much is bad-too little, too:
Cells need the proper fuel.

Inside the cell sugar's stored
Glycogen the sweetest hoard.
Two enzymes live to break it down:
A neutral enzyme can be found

In cytosol where granules roam,
But in the acidic lysosome
Another waits on bended knee
To play its role in glycophagy.

Excess carbs are bad: this much is clear.
So consider maltose when quaffing beer!

Roberta A. Gottlieb

Mitochondrial Mysteries

We know so much yet understand so little
About mitochondrial ox-phos and fusion and fission,
Mitochondrial autophagy and biogenesis.
MitoTimer and lenses have given us celluvision.
Though heart cells live years it's quite different within:
Mitochondrial life is counted in weeks.
Outer and inner membrane proteins vary yet more
In their lifespans revealed by mass spectrum peaks.
Protein import must match what's inside,
Lest proteins unfold and fall prey to Lon.
The peptides escape to the cytosol
To trigger transcription of chaperones.
Try we must to describe and define
The complex nature of the proteome,
As mitochondria expand and divide,
Fragment and fall into autophagosomes.
Yet for all we know and all we learn
The mysteries grow and questions expand
Like Mandelbrot sets of fractal images,
We see the work of divinity's hand.

Roberta A. Gottlieb

The Autophagy Process

A photo is static, an instant in time,
Telling nothing that happened next or before;
Yet papers are published and conclusions are drawn
Claiming autophagy's up when really it's gone.

Like tea leaves or runes it's not easy to read
When puzzling out blots of LC3B
When chloroquine's there or when it's left out
It's the increase that matters to tell you what's what.

Beclin is tricky when it's gone half away,
Its effects on autophagy go every which way:
Nucleation is up or fusion's not seen,
So consider with care what it all means.

Like freeways with cars and crowded on-ramps
AVs have cargo and their own traffic jams
Created by leupeptin, CQ or Baf,
LC3 rises at least by a half.

Remember when calculating if flux is intact
It's the ratio that gives you that crucial fact
LC3 levels of Baf over static
Will yield results that are not so erratic.

To understand the process you must think it through;
Autophagy requires you to use every clue.
Good data help you line up ducks,
Just please be sure to measure flux.

Roberta A. Gottlieb

Foreword

It is my pleasure to present Volume 8 of this impressive series on Autophagy edited by M. A. (Eric) Hayat. This volume begins with the latest developments in our understanding of the molecular mechanisms of autophagy, particularly the function of Beclin-1. There is an exponential increase in our recognition of the role of autophagy in many different diseases. This volume takes on cell death, mitochondrial diseases, protein misfolding, glycogen handling, neurodegeneration, as well as a comprehensive and contemporary series of chapters on cancer. Several chapters address the role of autophagy in the tumor microenvironment, cancer therapy, and its activation by various stimuli, including phytochemicals. These 17 chapters from authors from across the globe provide a detailed portrait of autophagy in health and disease, spanning mechanistic and clinical topics.

A more nuanced understanding of autophagy emerges when its beneficial and deleterious effects are considered in a single tissue responding to different stresses. New insights emerge from these chapters which address the contradictory consequences of autophagy activation or inhibition. Several chapters focus on mitochondria as special targets of autophagy and as major players in human disease, and the link between autophagy, mitochondrial dysfunction, and cell death is examined in several chapters, using different disease contexts to illustrate the complexity of the subject.

These topics are concisely presented in the well-articulated chapters of Volume 8. Researchers seeking to understand the contribution of autophagy to cancer and other diseases will appreciate the insights to be found in these chapters.

Roberta A. Gottlieb M.D.
Cedars-Sinai Heart Institute

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 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, called *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; the present book series also contributes to the exponential growth. Since the slow start after the discovery of the first autophagosomes by electron microscopy in the 1950s, autophagy has finally received the attention it deserves.

For a long time, autophagy was considered to be non-selective, 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 first molecular mechanisms and 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 the role of autophagy in disease. 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 autophagy. 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 the role of autophagy in disease and health.

Eeva-Liisa Eskelinen

Preface

Autophagy serves to maintain healthy cells, tissues, and organs, but also promotes cancer survival and growth of established tumors. Impaired or dysregulated autophagy can also contribute to disease pathogenesis. This is the eighth volume of the multivolume series, Autophagy: Cancer, Other Pathologies, Inflammation, Immunity, Infection, and Aging. This series discusses in detail almost all aspects of the autophagy machinery in the context of health, cancer, and other pathologies. Autophagy maintains homeostasis during starvation or stress conditions by balancing the synthesis of cellular components and their deregulation by autophagy.

Chapter 1 is the review of many aspects of the autophagy machinery, including its role in health and disease, including cancer. This chapter also contains information on protein synthesis and selective autophagy (allophagy, axonophagy, chromatophagy, ciliophagy, crinophagy, exophagy, glycophagy, lipophagy, lysophagy, mitophagy, nucleophagy, pexophagy, reticulophagy, ribophagy, xenophagy, and zymophagy). The mechanisms responsible for protein misfolding and refolding are explained, as complex post-translational modifications of proteins, including autophagy-related proteins, are a common phenomenon. Therefore, information regarding the conformational modifications of proteins is a prerequisite to fully understand the removal of unfolded, misfolded, incompletely folded, or aggregated proteins by autophagy or other mechanisms. Other chapters discuss in detail various aspects of autophagy. Selective autophagy degrades large intracellular aggregates and dysfunctional organelles. The failure of this mechanism is linked to neurodegenerative diseases. Pharmacological induction of autophagy has been suggested as a treatment for these disorders. Indeed, nutrient starvation activates autophagy. However, nutrient starvation-induced authophagy is detrimental to neurons under some circumstances.

The role of autophagy in disease, including cancer, is discussed in detail. Mitochondrial diseases are a group of rare genetic disorders caused by total or partial dysfunction of mitochondria. Mitophagy refers to the selective degradation of mitochondria by autophagy, which requires complementary factors such as low mitochondrial membrane potential and Parkin translocation to mitochondria. Both bulk autophagy and mitophagy have a protective role in mitochondrial diseases, but massive mitophagy can cause bioenergetics collapse of the cell. The structure and function of the high mobility group box 1 (HMGB1) are explained as this non-histone chromosomal protein regulates autophagy; for example, nuclear HMGB1 regulates heat shock protein β-1 expression, controlling mitochondrial quality control. The role of *EPM2A* and *NHLRC1* gene mutations in the Lafora disease is explained. The latest advances in the involvement of etiopathogenesis in Parkinson's disease are presented. An attempt is also made to clarify under which circumstances and to which extent inducers and inhibitors of autophagy are beneficial in clinical practice. Because Beclin

1 network plays a key role in controlling the mammalian autophagic process in response to physiological and pathological stress, its structure and function are detailed. It is explained that Sirt1 deacetylase regulates autophagy machinery via multiple mechanisms. The crosstalk between apoptosis and autophagy regulates cell survival or cell death in response to stress signals at cell or organism level.

Although autophagy is an important protective mechanism against a range of stress conditions, including starvation, cells undergoing cell death often display autophagic features, leading to the coining of the term, "autophagic cell death," the relevance of which is under debate. Therefore, it is important to discuss the recent advances regarding the role of autophagy in cell death in this volume.

The role of autophagy in cancer is exceedingly complex, and varies according to tumor type, stage, and genetic context. Regarding cell survival, tumor cells themselves activate autophagy in response to cellular stress and/or increased metabolic demands related to rapid cell proliferation. Autophagy-related stress tolerance can enable cell survival by maintaining energy production that can support tumor growth and therapeutic resistance. Tumors are often subjected to metabolic stress due to insufficient vascularization. Under these circumstances, autophagy is induced in cells located in these hypoxic regions where it enables their survival. Aggressive tumors have increased metabolic demands because of their rapid proliferation and growth and exhibit augmented autophagy needed for their survival. An attempt is made in this volume to explain the inter-conversation between cytoprotective and cytotoxic roles of autophagy in cancer. It is important to focus on the dual role of autophagy in tumorigenesis and chemotherapy, and discuss the future anti-cancer drug development. Based on the evidence that omega-3 DHA and EPA conjugates exert antiproliferative effects in breast cancer cells by inducing autophagy and apoptosis, the use of these conjugates as preventive agents against these cancer cells is recommended.

It is known that tumors hide from the immune response, and activate a counterattack or develop resistance to immune cells. It is explained in this volume that hypoxia-induced autophagy allows tumor cells to negate an effective immune response and escape from immune surveillance. It is suggested that autophagy represents a conceptual realm for new immunotherapeutic strategies aiming to block immune escape. Plant-derived chemicals (phytoagents) have become an important resource of therapeutic agents for cancer treatment. Several phytoagents that induce oxidative stress, stimulating protective autophagy, are discussed. This type II programmed cell death complements apoptotic cell death to enhance the killing of cancer cells.

By bringing together a large number of experts (oncologists, physicians, medical research scientists and pathologists) in the field of autophagy, it is my hope that substantial progress will be made against the terrible diseases that afflict humans. It would be nigh impossible for a single author to cover the current state of knowledge of this exceedingly complex process of autophagy. The participation of multiple authors allows for the presentation of different points of view on controversial aspects of the role of autophagy in health and disease.

This volume was written by 52 contributors representing 14 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 role of autophagy in disease provided by these contributors.

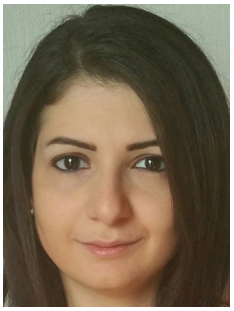
It is my hope that subsequent volumes of this series will join this volume in assisting in the more complete understanding of the complex process of autophagy and eventually in the development of therapeutic applications. There exists a tremendous urgent demand by the public and the scientific community 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. Roberta Gottlieb, M.D., for composing the poems that describe the process of Autophagy with appropriate humor and also for contributing the Foreword. I greatly appreciate thoughtful help extended to me by Linda Versteeg-Buschman, the senior editor at Elsevier, before and during the publication of this series. I offer thanks to Dr. Dawood Farahi and Phillip Connelly, for recognizing the importance of medical research and publishing through an institution of higher education. I am thankful to Elizabeth Shust and my students for their contributions to the final preparation of this volume.

June 2015
M. A. Hayat

Contributors

José A. Sánchez Alcázar Centro Andaluz De Biología del Desarrollo-CSIC, Universidad Pablo de Olavide, Sevilla, Spain



Tsolere Arakelian Laboratory of Experimental Cancer Research, Department of Oncology, Luxembourg Institute of Health, Luxembourg City, Luxembourg

Sebastiano Andò Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, Arcavacata di Rende, Italy

Guy Berchem Laboratory of Experimental Cancer Research, Department of Oncology, Luxembourg Institute of Health, Centre Hospitalier de Luxembourg, Department of Hemato-Oncology, Luxembourg City, Luxembourg

Daniela Bonofiglio Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, Arcavacata di Rende, Italy

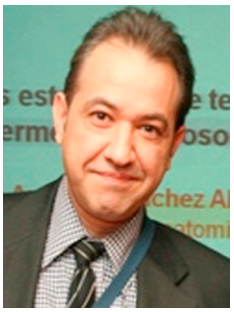
Patrice Codogno Institut Necker Enfants-Malades, Université Paris Descartes-Sorbonne Paris Cité, Paris, France

Sandra Morais Cardoso Center for Neuroscience and Cell Biology, Institute of Biology, Faculty of Medicine, University of Coimbra, Coimbra, Portugal

Mario D. Cordero Centro Andaluz De Biología del Desarrollo-CSIC, Universidad Pablo de Olavide, Sevilla, Spain

Stefania Catalano Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, Arcavacata di Rende, Italy

David Cotán Centro Andaluz De Biología del Desarrollo-CSIC, Universidad Pablo de Olavide, Sevilla, Spain



Mario de la Mata Centro Andaluz De Biología del Desarrollo-CSIC, Universidad Pablo de Olavide, Sevilla, Spain

Subramaniam Ganesh Department of Biological Sciences and Bioengineering, Center of Excellence for Chemical Biology, Indian Institute of Technology, Kanpur, India

Isabel de Lavera Centro Andaluz De Biología del Desarrollo-CSIC, Universidad Pablo de Olavide, Sevilla, Spain

Marc Germain Medical Biology Department, Université du Québec à Trois-Rivières, Québec, Canada

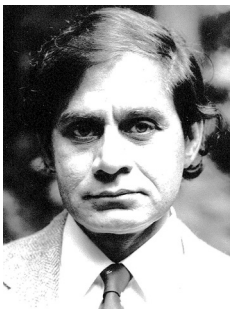
Ana Raquel Esteves CNC-Center for Neurosciences and Cell Biology, University of Coimbra, Coimbra, Portugal

Cinzia Giordano Health Center, University of Calabria, Arcavacata di Rende, Italy

Rui Gomes Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal

Gérald Guillebaud Medical Biology Department, Université du Québec à Trois-Rivières, Québec, Canada

Elisabet Alcócer Gómez Centro Andaluz De Biología del Desarrollo-CSIC, Universidad Pablo de Olavide, Sevilla, Spain

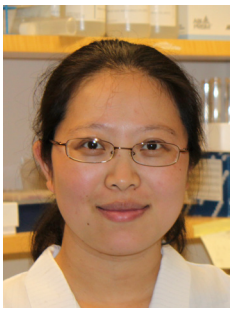


Eric Hayat Department of Biological Sciences, Kean University, Union, NJ, USA

Maria Gonçalves-Fernandes Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal

Gu He State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, Sichuan University, Collaborative Innovation Center of Biotherapy, Chengdu, China

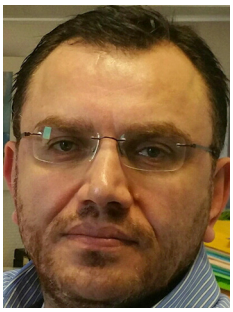
Zhaoyue He Institute of Pharmacology, University of Bern, Bern, Switzerland



Rui Kang Department of Surgery, University of Pittsburgh Cancer Institute, Pittsburgh, PA, USA

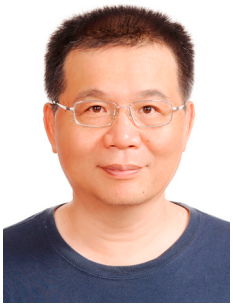
Ming-Shiou Jan Institute of Microbiology and Immunology, Division of Allergy, Immunology, and Rheumatology, Medical College of Chung Shan Medical University, Taichung, Taiwan

Munehiro Kitada Department of Diabetology and Endocrinology, Kanazawa Medical University, Ishikawa, Japan



Bassam Janji Laboratory of Experimental Cancer Research, Department of Oncology, Luxembourg Institute of Health, Luxembourg City, Luxembourg

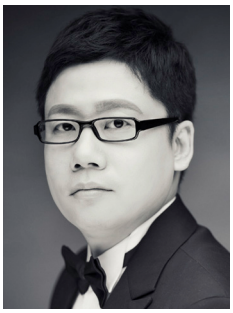
Daisuke Koya Department of Diabetology and Endocrinology, Kanazawa Medical University, Ishikawa, Japan



Jung-Hua Steven Kuo Department of Pharmacy, Chia Nan University of Pharmacy and Science, Tainan, Taiwan

Chia-Wei Lin Institute of Microbiology and Immunology, Medical College of Chung Shan Medical University, Taichung, Taiwan

Marilena Lanzino Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, Arcavacata di Rende, Italy



Bo Liu State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, Sichuan University, Collaborative Innovation Center of Biotherapy, Chengdu, China

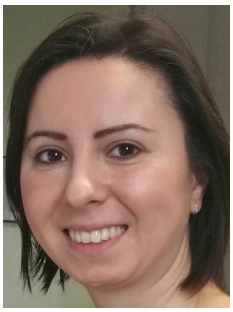
Wai-Leng Lee School of Science, Monash University Malaysia, Selangor Darul Ehsan, Malaysia

He Liu Institute of Pharmacology, University of Bern, Bern, Switzerland

Shouqing Luo Centre for Biomedical Research-Translational & Stratified Medicine, Peninsula School of Medicine and Dentistry, Plymouth University, Plymouth, UK

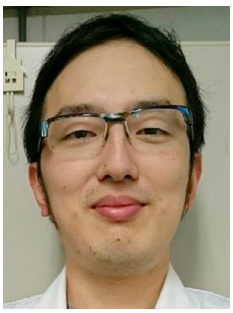
Sandrine Medves Laboratory of Experimental Cancer Research, Department of Oncology, Luxembourg Institute of Health, Luxembourg City, Luxembourg

Yuting Ma Apoptosis, Cancer & Immunity Laboratory, INSERM Cordeliers Research Center, Université Paris Descartes, Paris, France



Takouhie Mgrditchian Laboratory of Experimental Cancer Research, Department of Oncology, Luxembourg Institute of Health, Luxembourg City, Luxembourg

Juan Garrido Maraver Centro Andaluz De Biología del Desarrollo-CSIC, Universidad Pablo de Olavide, Sevilla, Spain



Yoshio Ogura Department of Diabetology and Endocrinology, Kanazawa Medical University, Ishikawa, Japan

Kan-Fu Oh School of Science, Monash University Malaysia, Selangor Darul Ehsan, Malaysia

Marina Villanueva Paz Centro Andaluz De Biología del Desarrollo-CSIC, Universidad Pablo de Olavide, Sevilla, Spain

Manuel Oropesa-Ávila Centro Andaluz De Biología del Desarrollo-CSIC, Universidad Pablo de Olavide, Sevilla, Spain

Shensi Shen Institut Gustave Roussy, Université Paris Sud, Villejuif, France

Ana Delgado Pavón Centro Andaluz De Biología del Desarrollo-CSIC, Universidad Pablo de Olavide, Sevilla, Spain

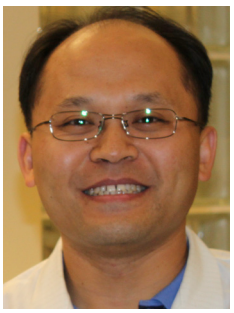
Diana Filipa Silva CNC-Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal

Hans-Uwe Simon Institute of Pharmacology, University of Bern, Bern, Switzerland

Elodie Viry Laboratory of Experimental Cancer Research, Department of Oncology, Luxembourg Institute of Health, Luxembourg City, Luxembourg

Pankaj Kumar Singh Department of Translational Medicine and Neurogenetics, Institut de Génétique et de Biologie Moléculaire et Cellulare, Illkirch, France

Yangchun Xie Department of Surgery, University of Pittsburgh Cancer Institute, Pittsburgh, PA, USA



Daolin Tang Department of Surgery, University of Pittsburgh Cancer Institute, Pittsburgh, PA, USA

Peng-Nian Yew School of Science, Monash University Malaysia, Selangor Darul Ehsan, Malaysia

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M.A. *Hayat*

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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 autophagosomes, 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 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, taking regular exercise and sleep, 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, 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 the prevention and therapy of disease processes, autophagy has emerged as a potential target for disease.

Unfortunately, inevitable death rules our lives. It is known that there is "no birth in the absence of death and vice versa"; a group of abnormal cells plays a part in this process. 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 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 these 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 distinct intracellular and extracellular milieu of lysosomes. 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.

During 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 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 reactive oxygen species (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, and 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 against 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, prodegradation or antidegradation of pathogens, sequestration of damaged lysosomes, cellular signaling, maintenance of muscle mass, homeostatic maintenance of adult tissues, tissue remodeling, neuronal degeneration, survival from deprivation of nutrition and growth factors, prevention of malignant transformation, degradation of excessive and dysfunctional cellular components, prevention or delaying the growth of transformed normal cells, association with apoptosis and mitochondrial change in pathogenesis, induction of osteogenesis, antigen presentation, chromatophagy, nucleophagy, depending on the cell/tissue context: modulation of cell migration and membrane recycling, and other functions.

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.

ENDOPLASMIC RETICULUM

All eukaryotic cells contain an endoplasmic reticulum (ER), and its highly convoluted single membrane typically constitutes more than half of the total membrane system of the cell. Ribosomes are attached to the surface of the rough ER membranes, but ribosomes are also found free in the cytosol. These two types of ribosomes are the site of synthesis of different classes of proteins.

ER plays a central role in cell biosynthesis. The synthesis of transmembrane proteins and lipids of the ER, Golgi complex, lysosomes, and plasma membrane begins in association with the ER membrane. Most of the lipids that constitute the membranes of mitochondria and peroxisomes are also contributed by the ER. In addition, all of the newly-synthesized unfolded proteins are first delivered to the ER lumen for refolding before becoming part of the Golgi complex and lysosomes. Disulfide isomerase and chaperone Hsc70 proteins catalyze the refolding. ER is also involved in the synthesis of secreted proteins and formation of the extracellular matrix. Indeed, ER is the center of chaperone proteins that are responsible for correct folding of secreted proteins. In this system, lectin-binding proteins (calreticulin

and calnexin) facilitate glycoprotein folding; glucose regulated protein complex is also involved in this system (McLaughlin and Vandenbroeck, 2011).

Another important function of ER, as indicated above, is in the biogenesis of autophagosomes by providing the site for omegasome formation and the source of membrane used. Double FYVE domain-containing protein 1 (DFCP1) is also located at ER and Golgi membranes instead of endosomes, and is involved in the formation of autophagosomes. This protein contains two FYVE domains, explaining its PI(3)P binding. Ave et al. (2008) have exploited the localization and movement of DFCP1 during amino acid starvation for identifying a PI(3)P-enriched compartment dynamically connected to the ER. It was further demonstrated that a PI(3)P compartment was formed near the VPS34-containing vesicles that provide a membrane platform for the accumulation of autophagosomal proteins, expansion of autophagosomal membranes, and fully formed autophagosomes.

Eukaryotic cells are exposed to a large variety of cellular stresses, including nutrient or growth factor deprivation, hypoxia, reactive oxygen species, DNA damage, protein accumulation, and damaged cell organelles. These cells must also adapt to functions in parameters such as temperature, ultraviolet light, ion concentrations, pH, oxygen tension, redox potentials, hormones, cytokines, and neurotransmitters (Kroemer et al., 2010).

Most of the folding and posttranslational processing of membrane-bound proteins and secreted proteins occur in the ER under an array of chaperone systems such as glycosidases, Ca^{2+}-dependent chaperones, and members of the protein disulfide isomerase family (Deegan et al., 2013). These chaperones are responsible for the correct folding of proteins under normal physiological conditions. When this finely balanced unique environmental condition is disrupted, the protein folding machinery of the ER becomes less efficient or nonfunctional, resulting in the ER stress.

It has been reported that human hepatitis B virus may induce autophagy through the induction of ER stress (or through the HBx protein) enhancing its replication (Tian et al., 2015). The involvement of ER stress in Crohn's disease has also been reported (Bringer et al., 2014). ER stress, in addition, is known to induce autophagy-mediated cell death, and DAP (death-associated protein kinase) is an essential mediator of this process (Kabi and McDonald, 2014).

The ER stress and ubiquitin–proteasome system (UPS) are intimately intertwined. The ER stress is capable of activating autophagy, a function that is conserved from yeast to mammals (Fritz et al., 2013). It is known that eukaryotic initiation factor 2 induces autophagosome formation under stress conditions (Ogata, 2006). Thus, autophagy complements ER-associated degradation (ERAD) induced during unfolded protein response (UPR) (Fujita, 2007).

It is now accepted that when cell is subjected to stress, depending on the amount of stress, the autophagy can induce either a cell survival signal or a death signal. If the stress is mild, it generates an adaptive response to survival against the stress, and autophagy results in survival signal by activating several genes and transcription factors, which alter the stress-induced death signal into a survival signal (Das, 2011). This change leads to the production of antiapoptotic (antideath) proteins. In contrast, if the stress is excessive, the adaptive response fails, and the cell dies as a result of the introduction of apoptotic signals.

ENDOPLASMIC RETICULUM STRESS

The ER stress results when unfavorable physiological or pathological conditions cripple the ER protein folding machinery to correctly fold nascent proteins. It occurs upon the accumulation of misfolded or unfolded proteins in the ER. As a result, ER plays a pivotal role in the development of pathology of many neurodegenerative disorders, including Alzheimer's, Parkinson's, and prion (Creutzfeldt Jacob's disease) and amyotrophic lateral sclerosis.

DFCP1 during amino acid starvation for identifying a P1(3)P-enriched compartment dynamically connected to the ER. It was further demonstrated that this compartment was formed near the Hps34-containing vesicles that provide a membrane platform for the accumulation of autophagosomal proteins, expansion of autophagosomal membranes, and fully formed autophagosomes.

ER is also involved in the degradation (removal) of misfolded proteins. For example, Cortes et al. (2013) have demonstrated the intracellular trafficking and degradation of newly synthesized misfolded/aggregated mutant prion protein (PrP) assisted by ER.

The initial and rapid response of cells to the ER stress is the activation of a set of pro-survival signaling pathways called the UPR, which is a catabolic process resulting in autophagy and cell death (Doyle et al., 2011; Gorman et al., 2012). The primary function of UPR is to sustain cell survival.

The UPR regulates protein folding capacity of the ER by sensing the presence of unfolded proteins in the ER lumen, transmitting the information to the cell nucleus, where it drives a transcriptional program focused to reestablish homeostasis (Bernales et al., 2006b). In mammals, the six major ER sensors are: (1) IRE1 (inositol requiring 1), (2) IERN1 (ER-to-nucleus signal: img 1), (3) PERK (double-stranded RNA-activated protein kinase (PKR)-like kinase), (4) PEK (pancreatic eukaryotic initiation factor 2α kinase), (5) EIF2AK3, and (6) ATF6 (activating transcription factor 6) (Ron and Walter, 2007). IRE1 and PERK are type 1 transmembrane proteins with protein kinase activity, while ATF6 is a type II transmembrane protein encoding a transcription factor (Schröder and Kaufman, 2005). Bernales et al. (2006a,b) demonstrated that the ER volume increased under UPR-inducing conditions in the yeast. The ER expansion was accompanied by the formation of autophagosomes that packed membranes derived from the UPR-expanded ER. The ER-specific autophagy utilizes autophagy genes. Such genes are activated by the UPR and are essential for the survival of cells exposed to ER stress. Such selective ER sequestration maintains a steady-state level of ER abundance during continuously accumulating unfolded proteins (Bernales et al., 2006a).

The UPR also blocks protein synthesis and activates mechanisms that prepare the cell to cope with the aggregated unfolded proteins. One of such mechanisms involves the enhancement of the protein folding capacity of the ER by increasing the expression of ER chaperone proteins and up-regulating the degradation of misfolded proteins (Doyle et al., 2011). However, prolonged or excess ER stress may activate apoptosis. Pro-apoptotic factors (including cytochrome c) are released by the UPR by opening the mitochondrial permeability transmembrane pores. In conjunction with apoptotic protease activating factor 1, pro-caspase 9, and cytochrome c form the apoptosome (Olson and Kornbluth, 2001). The apoptosome is a complex consisting of adaptor proteins that mediate the activation of initiator caspases at the onset of apoptosis.

In conclusion, the development of the UPR protects cells from the deleterious effects of the ER stress. When the ER stress is not removed, it can be lethal or harmful to cells causing neurodegenerative and cardiovascular diseases, cancer, or diabetes. Overexpression of Bcl-2 also protects cells from ER stress-induced death. Conditions that induce ER stress also induce autophagy. It is well-established that autophagy constitutes a major protective mechanism that allows cells to survive in response to multiple stressors, and it helps organisms to defend against degenerative, inflammatory, infectious, and neoplastic disorders. It needs to be noted that ER stress itself is capable of activating autophagy, while impaired autophagy can promote ER stress.

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 ubiquitination (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, similarly 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 autophagosomes, 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 toward 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, along with its ribosomes, it 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 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.

PROTEIN SYNTHESIS

It is known that autophagy plays a key role in the degradation of aberrant proteins. It is also known that if such proteins are not correctly refolded or degraded, they tend to accumulate, resulting in neurodegenerative and other diseases. Misfolded proteins result from mutations, for incomplete translation gives rise to defective ribosomal products. The delivery of misfolded proteins to lysosomes for their degradation by autophagy requires the function of several protein complexes and pathways, including mTORC1 complex, ULK1/Atgl complex, LC3 conjugation pathway, and P13K class III/VPS34 complex (Wani et al., 2015).

It is also known that autophagic proteins and other proteins are subjected to regulatory posttranslational modifications. Proteins are slowly biosynthesized on the ribosome, and most of them do not begin to fold during synthesis and tend to form aggregates because of hydrophobic interactions (Figure 1.1). Phosphorylation is the most important posttranslational modification in the autophagy process, followed by ubiquitination and acetylation (Wani et al., 2015). Phosphorylation induces changes in protein configuration by inserting a phosphate group onto serine, threonine, and tyrosine amino acids, resulting in the activation of autophagy and autophagosome formation, which, in turn, depend on the direct phosphorylation of Atg9 by the Atg1 kinase (Papinski et al., 2014). It is known that most proteins contain modifiable serine, threonine, lysine, or cysteine residues in response to nutrient availability, growth factor deprivation, hypoxia, hyperoxia, and generation and

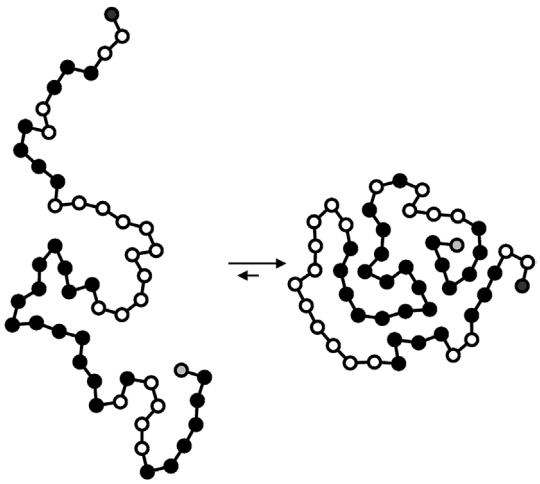

FIGURE 1.1 The main driving force responsible for the formation of protein structure. The diagram on the left side shows hydrophobic amino acids (black spheres) exposed to the external space, while the diagram on the right side shows hydrophobic amino acids buried inside, shielded from the solvent. When the hydrophobic amino acids are exposed to high temperatures, high concentration of solutes, and chemical denaturants, proteins may not fold into their biochemically functional form or may unfold. Under certain conditions, chaperones assist proteins both in proper folding and in remaining folded. Chaperones may also unfold misfolded proteins and provide them with a second opportunity to refold properly.

propagation of reactive species (Wani et al., 2015). Based on this and other evidence, it is apparent that a better understanding of the synthesis of proteins, especially misfolded (incompletely) proteins, is needed to decipher the molecular mechanisms underlying protein accumulation, sequestration, and degradation.

It is important to know the fundamental molecular mechanisms underlying the formation of normal and abnormal proteins for understanding the function or failure of function of both types of proteins. Indeed, in order to understand the necessity of the removal of unfolded, misfolded, incompletely folded, or aggregated proteins by autophagy or other mechanisms, knowledge of the formation of such proteins is needed. Information regarding the conformational modifications of proteins (glycoproteins) is a prerequisite to fully understand this necessity. The removal of aberrant proteins avoids invitation to some pathological conditions such as neurodegenerative diseases. This subject is discussed in detail below.

The word protein is derived from the Greek word *proteios*, meaning first or foremost, reflecting the functional importance of this molecule. Proteins participate directly or indirectly in virtually every process in a cell. Most of the chemical reactions in and structural components of a cell are mediated or supplied by proteins. In other words, proteins have many kinds of functions: structural proteins, regulatory proteins, and catalytic proteins. In addition to generating these biological activities, protein molecules are coupled to many other biological processes, including trafficking. Proteins are constantly synthesized as cells grow, reproduce, and repair themselves. So, proteins must be replenished during the life of the cell, which is carried out by autophagy and other processes. Proteins are long polymers made out of 20 amino acids. A protein of an average size has 300 amino acid residues, and the possible combinatorial number of proteins made with 20 amino acids is enormous. Amino acids are added sequentially instead of randomly to form a correct polypeptide under normal physiological conditions. The polypeptide always begins at its N-terminus and not its C-terminus. A polypeptide may have a length of approximately 150 nm. Polypeptides are encoded by genes in the DNA.

All the information required for a protein molecule to fold into its three-dimensional conformation is contained in the amino acid sequence. The three-dimensional structure of a protein allows it to perform its function, to connect with reactive sites on other proteins and molecules within the cell. Amino acids possess side chains, and some of them are hydrophobic, while others are hydrophilic; some are positively charged, while others are negatively charged. In a properly folded protein molecule, hydrophobic amino acids are located inside the protein molecule, whereas hydrophilic amino acids are located on the surface of the protein (Figure 1.1). In contrast, in a misfolded protein, hydrophobic amino acids are located on the surface of the molecule, inviting chaperones to help correctly refold misfolded or partly folded proteins.

Knowledge of the number of protein domains, which show little or no ordered structure under physiological conditions, has been increasing exponentially during the last two decades. Natively unfolded proteins lack ordered structure under conditions of neutral pH *in vitro*. These proteins are specifically located within a unique region of charge hydrophobicity phase space, and a combination of low overall hydrophobicity and large (high) net charge represents a unique structural feature of natively unfolded proteins (Uversky et al., 2000). Maintaining quality control over protein structure and function depends on molecular chaperones and proteases, both of which can recognize hydrophobic regions exposed on

unfolded polypeptides (Wickner et al., 1999). Molecular chaperone proteins and the ubiquitin-proteasome degradation pathway protect eukaryotic cells against buildup of misfolded proteins; the former assist folding of newly translated and stress-denatured proteins (Hartl and Hayer-Hartl, 2009). Nevertheless, some misfolded polypeptides are not folded correctly under any circumstance (Zhang and Qian, 2011).

Cellular proteins can be correctly folded (native state), misfolded, unfolded, incompletely folded, or aggregated. Figure 1.2 shows schematic configurations of typical polypeptides, misfolded, aggregated protein molecules, and three-dimensionally-folded, normal protein. The correct native interactions are more favorable than the incorrect nonnative ones.

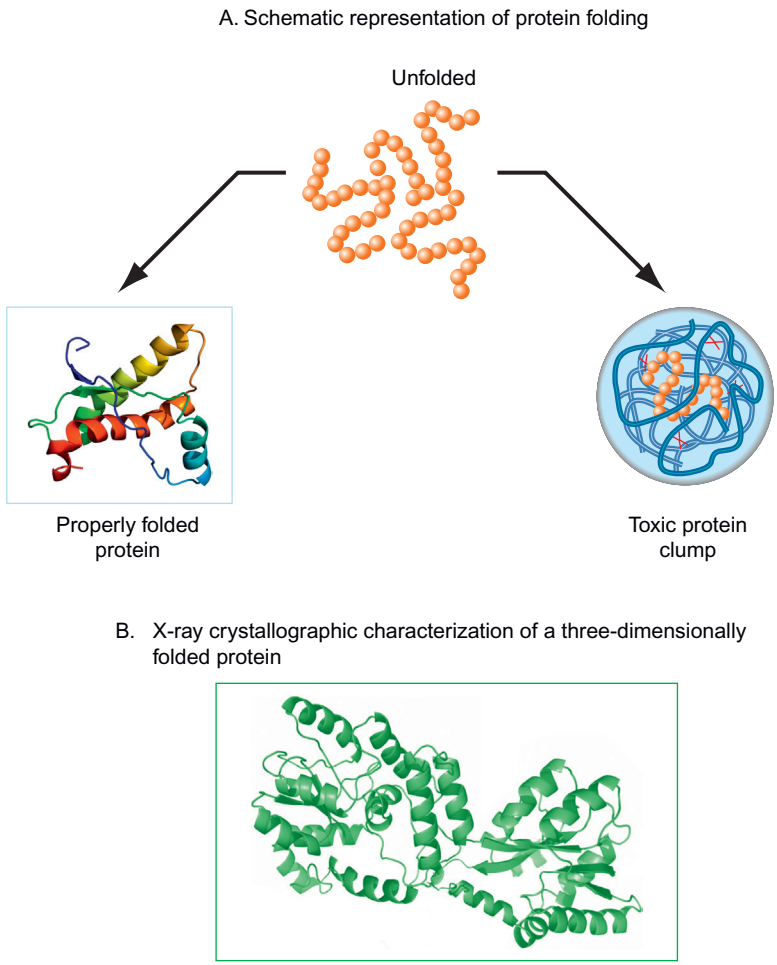

FIGURE 1.2 Protein folding. (A) Schematic representation of protein folding. (B) X-ray crystallographic characterization of a three-dimensionally-folded protein.

However, even the most carefully designed polypeptide may misfold and find itself in a nonnative state where it might be at least transiently stable (Dobson, 2014). Only correctly folded proteins have long-term stability in a crowded biological environment to be able to interact selectively with their natural partners that include DNA, RNA, other proteins or peptides, and membranes. Thus, failure of proteins to fold correctly or to remain incorrectly folded results in a wide variety of pathological conditions, including neurodegenerative diseases. In addition to the mechanism underlying the formation of aberrant proteins already discussed earlier in this chapter, the aging process is accompanied by mutations and thermodynamics, which conspire against us, resulting in the misfolding or incomplete folding of proteins (Renaud, 2010).

Proper protein folding in the ER lumen is associated with the formation of disulfide bonds that are covalent linkages between two sulfhydryl groups in the two adjacent cysteine side chains. These bonds help to reinforce the conformation of the protein, stabilizing its structure. The presence of ATP, CA^{2+} concentration, results in the minimization of protein folding capacity of the ER (Gorman et al., 2012). Consequently, unfolded proteins accumulate in the ER, causing ER stress (see later). However, the development of the UPR protects the cells from the deleterious effects of the ER stress.

As mentioned above, formation of disulfide bonds is essential for the proper folding of proteins. In eukaryotic cells, disulfide bonds are confined to proteins synthesized in the ER. The reducing environment of the cytosol is not conducive to the formation of disulfide bonds, and thus they are formed in the ER. The ER lumen contains the enzyme protein disulfide isomerase (PD1) which catalyzes the formation of disulfide bonds between cysteine residues. Cysteine residues in their active site help to catalyze this reaction. This process precedes the completion of synthesis of newly forming polypeptides.

As expected, PD1 becomes reduced in this process and needs to be reoxidized to be able to participate in another round of oxidation. This reaction depends on the ER protein oxidase 1 (Ero1) which contains Flavin adenine dinucleotide as a prosthetic group. Ero1 also contains a disulfide bond that is used to regenerate oxidized PD1. Ero1 itself is oxidized by molecular oxygen. In addition to forming disulfide bonds, PD1 can catalyze rearrangement of disulfide bonds, facilitating the enzyme to correct any inappropriate disulfide bonds that may have been formed as a protein folds. In this case, reduced PD1 forms a disulfide bond with the protein.

The role of water molecules has been suggested to influence protein folding, instead of tumbling among themselves. Water molecules in the cell form teeming, shifting shells around ions or sleeves around biomolecular chains. Water molecules may erect invisible, stability-enhancing scaffolds. Intracellular water molecules may assist biomolecules in their search for binding partners. Water molecules may form hydration funnels surrounding the binding pockets of proteins (Ruhr University Bochum). These funnels seem to facilitate biomolecules to recognize and bind to each other, such as correct protein folding.

It does not seem to be uncommon that the structure and shape of a protein molecule undergo changes during its lifetime, depending upon its immediately required function. This phenomenon is fascinatingly shown by the HIV spike protein. A recent study by Munro and Mothes (2015) indicated that this protein rapidly shifts three different configurations, one of which is a closed state that is inaccessible to antibodies. Subsequently,

it reverts to an open state to fuse and infect cells. Indeed, these transformations allow the virus to resist the immune defense system but also can infect cells. Apparently, an effective vaccine needs to be based on the closed state form of this viral protein. These authors obtained this information by using single-molecule fluorescent resonance energy transfer microscopy at an atomic resolution. Finally, it is recognized that biological macromolecules are far from being rigid in their structure. Motion is also implicit in the normal function of molecules such as serum albumin and myoglobin (Austin et al., 1975). Binding sites for interacting proteins are frequently more mobile than the rest of the protein (Tainer et al., 1984).

Cells carry out their various functions by synthesizing and degrading proteins on a regular basis. The routine degradation of abnormal (misfolded) and unwanted (excessive) proteins is accomplished primarily by the UPS. The presence of unfolded proteins triggers the repair of these proteins by activating chaperone proteins. If this system fails, the cell activates the autophagy degradation mechanism to degrade these abnormal proteins. If this mechanism is not fully effective, misfolded proteins form aggregates (aggresomes). The formation of aggresomes may lead to autophagy. An aggresome is a protein complex containing abnormal proteins, chaperone proteins, proteasome components, mitochondrial components, and ubiquitinated proteins. The protein aggregates are transported along the microtubule network by motor protein dynein (Mi et al., 2009). These authors also reported that isothiocyanate (ITC) (a small cancer chemopreventive molecule) can induce formation of aggresome-like structures.

Methods

A number of methodologies are available to study protein structure, unfolding, and refolding, three of them are summarized here. Uversky et al. (2000) have presented a method to predict whether a given protein assumes a defined fold or is intrinsically unfolded. This method is based on the average hydrophobicity of its amino acids and the value of its net charge. This simple procedure facilitates rapid prediction of whether a given amino acid sequence is disordered or not. These authors have presented a list of 91 known natively unfolded proteins and their major characteristics. The number of residues in these proteins ranges from 50 to 1827, and the net charge at pH 7.0 may range from +59 to –117.

The second method consists of atomic force microscopy (AFM) which has become a prominent tool for studying the mechanical properties of proteins and protein interactions on a single-molecule level using chemicals or temperature as a denaturant (Borgia et al., 2008). The effect of force on thermodynamics and kinetics of single molecule reactions can be studied (Tinoco and Bustamante, 2002). Because many proteins fulfill mechanical functions or exert mechanical forces in their natural environment, AFM facilitates targeting physiologically relevant questions. Another method in use is nuclear resonance microscopy that is particularly applicable to the study of unfolded and partly folded proteins. It provides unique structural insights into the events of protein folding process (Dyson and Wright, 1996, 2004, 2005). This approach has been instrumental in identifying and characterizing functional domains of these proteins.

ABNORMAL PROTEINS

Intracellular proteins are subjected to continuous turn-over through coordinated synthesis, degradation, and recycling of their component amino acids. Proteins can undergo degradation by the proteasome or by lysosomes. Proteins are degraded by macroautophagy, microautophagy, or CMA. CMA is especially efficient in the degradation of damaged or abnormal proteins, fulfilling its role in quality control. However, proteolytic systems in certain cases fail to adequately dispose of deleterious proteins, which results in protein aggregation and neuronal demise causing neurodegenerative diseases.

The presence of unfolded or misfolded proteins in cells is not uncommon. It is estimated that approximately 30% of newly synthesized proteins are unfolded or incorrectly folded. The reason seems to be that protein folding is an exceedingly complex process because the transition from a linear sequence of amino acids to a correctly fully folded, three-dimensionally active protein requires at least favorable physiological environment and assistance from other biological molecules. The difference in the configuration of an unfolded polypeptide and a folded three-dimensional protein molecule is shown in Figure 1.3. It is known, for example, that low-molecular-weight chemical chaperones stabilize a protein as it folds into the proper structural form (Ferreon et al., 2012).

In order to understand the damage (e.g., Alzheimer's disease) caused by the accumulation of unfolded or misfolded proteins, it is important to identify and measure the quantity of such proteins. It is relevant to determine how much misfolded proteins actually cause cell damage or cell death. One method to visualize the interplay between fully folded and unfolded forms of proteins is by using a designed fluorescent-tagged small molecule (folding probe) (Liu et al., 2014). This probe specifically binds to the folded, functional protein, but not to misfolded forms of protein. Thus, the quantification method can determine the comparative amount of folded protein versus misfolded protein in a cell.

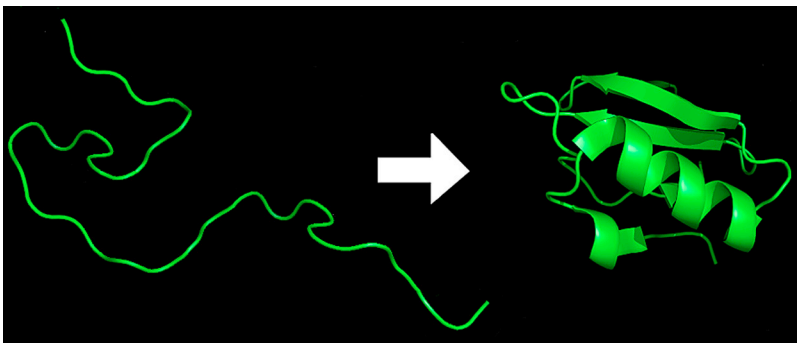

FIGURE 1.3 An unfolded polypeptide containing a linear chain of amino acids (left-hand side) and a three-dimensional structure of the folded protein (native conformation) (right-hand side). Unfolded or misfolded proteins either remain inactive or become functionally toxic as accumulation of such proteins (e.g., amyloid fibrils) may cause neurodegenerative diseases, allergies, or type 2 diabetes (proteopathies). A protein molecule may fold spontaneously during or after biosynthesis and/or its folding depends on the solvent (water or lipid bilayer), the salt concentration, the pH, the temperature, cofactors, and molecular chaperones.

Autophagy in most cases is able to degrade misfolded proteins. Information to correct protein misfolding is available. In certain cases, specific molecules (pharmacoperones) can correct protein misfolding in cells. An example of such therapeutic effect was reported by Janovick et al. (2013). They reported the rescue and expression of a misfolded G-protein-coupled receptor (hormone), which contained a single amino acid change; a negatively charged glutamic acid was substituted by a positively charged lysine. This modification resulted in the misfolding and misrouting of the gonadal protein (GnRHR). By using 1N3 (a small molecule), they accomplished proper folding of the misfolded protein and restored normal gonadal function in the mutant mice. The normal function resulted from correct routing of the protein to the plasma membrane instead of to the ER. It also became clear that misfolded protein was forming oligomers with wild-type GnRHR protein, effectively rendering the latter useless and a target for the quality control machinery of the cell. It is concluded that small molecules (e.g., 1N3) can be tried for the treatment of genetic diseases associated with misfolded proteins.

A different type of autophagy protein, intrinsically disordered or unstructured protein, is discussed below. Some autophagy proteins (Atgs) have intrinsically disordered regions (IDRs) and are called IDRPs, which are predicted in approximately 30% of the prokaryotic proteins and approximately 47% of eukaryotic proteins (Dunker et al., 2008). IDRPs have negligible folded tertiary structure or stable secondary structure elements such as α-helix and β-sheets. The importance of the IDRs in cellular processes has been overlooked, as biological roles and mechanisms of most of these regions are poorly understood. These regions play an important role in autophagy, and this role has not been adequately investigated.

IDRPs in contrast to Atgs are poorly conserved. IDRs seem to have diverse functions in different homologs. Recent studies indicate that IDRs facilitate protein–protein interactions (Mei et al., 2014). The importance of this role becomes apparent considering that many or even most Atgs function via formation of multi-protein complexes. These complexes initiate autophagy, autophagosome nucleation, and autophagosome expansion, maturation, and fusion with lysosomes.

Potential protein partners that might interact with the disordered regions have been identified (Mei et al., 2014). For example, a Bc12 homology 3 domain (BH3D) (within the key autophagy Beclin 1 protein) is an IDR. BH3D undergoes a conformational change from coil to α-helix upon binding to Bc12. The C-terminal half of this BH3D constitutes the binding motif, which serves to anchor the interaction of the BH3D to Bc12. Finally, the high preponderance of IDRs in autophagy proteins implies that these regions play a significant role in the autophagic functions. It needs to be noted that mutations implicated in major diseases, including cancer and neurodegenerative and cardiovascular disorders, map to IDRs (Uversky et al., 2008).

MOLECULAR CHAPERONES

Molecular chaperones (heat shock proteins (Hsps)) help misfolded proteins to correctly fold by binding to their hydrophobic surfaces. The chaperones are called Hsps because they are synthesized in increased amounts after cells are briefly exposed to high temperatures or any other types of stress (e.g., ER stress). Elevated temperatures cause an increase in

misfolded proteins, which, in turn, results in a feedback system that boosts the synthesis of chaperones, helping the misfolded proteins to refold. The best relevant eukaryotic chaperones are Hsp60 and Hsp70. Different members of the chaperone family function in different organelles and locations; for example, mitochondria contain their own Hsp60 and Hsp70 molecules, which differ from those that function in the cytosol.

A large number of chaperone proteins, foldases, and cofactors are expressed at the ER to promote correct folding and prevent abnormal aggregation of misfolded proteins. The existence of chaperones implies that some proteins have inherently unstable conformation that can change from a functional minimal-energy state to a state that is nonfunctional or even toxic. One of the most abundant chaperones in the ER lumen is a protein called Bip (binding protein), which is a member of the Hsp70 family of proteins. Bip binds to hydrophobic regions of the polypeptide chain, especially those enriched in the amino acids tryptophan, phenylalanine, and leucine. In contrast, in a correctly folded protein, such hydrophobic regions are aggregated and buried in the interior of the protein molecule and inaccessible to Bip. Bip recognizes newly synthesized proteins as they are translocated in the ER and maintains them in a state of competent for subsequent folding and oligomerization. Although chaperones help unstable proteins to fold correctly, some proteins remain misfolded and may form long linear or fibrillary aggregates known as amyloid deposits, including those in Alzheimer's disease.

THE ENDOPLASMIC RETICULUM

The ER is the main compartment where most of the proteins are synthesized and folded. Free ribosomes (not attached to the ER) are also involved in the synthesis of some proteins. Free ribosomes are especially active in synthesizing proteins that are retained within the cell, while ribosomes of the ER synthesize mainly proteins that are exported from the cell. Most of the proteins synthesized on ER ribosomes are glycoproteins. The glycosylation takes place in the ER while the growing polypeptide chain is still being synthesized. A typical eukaryotic cell has its own unique set of proteins in billions, representing at least 10,000 different kinds of polypeptides, each of these proteins finds its way either to the appropriate location within the cell or out of the cell.

The cell has quality control mechanisms that ensure that proteins are folded into their correct three-dimensional conformation before they can move from the ER to the appropriate destination in the cell (protein trafficking). Misfolded proteins are often eliminated by the quality control mechanisms after initially being retained in the ER instead of being immediately eliminated. The retention of misfolded proteins in the ER interrupts their proper trafficking, and the resulting reduced biological activity can lead to impaired cellular function, and ultimately to disease.

An interesting recent study by Cortes et al. (2013) demonstrated the intracellular trafficking and the degradation of newly synthesized misfolded/aggregated mutant PrP. They found that autophagy plays a key role in delivering such proteins from ER to lysosomes. In the absence of such quality control mechanism to limit the accumulation of misfolded PrP may lead to the generation of a pathologic misfolded isoform (PrPsc) causing genetic prion disease. This concept is strengthened by the finding that in normal cells treated with the autophagy inhibitor 3-MA, mutant-Prp colocalization with lysosomes is reduced.

Additionally, mut-PrP expression is associated with an elevation of several markers of the autophagy–lysosomal pathway and is extensively localized with the autophagic-specific marker, LC3B.

The accumulation of misfolded proteins in the ER may lead to various types of stress on cells, which may contribute to cellular dysfunction and disease such as neurodegenerative conditions. As stated earlier in this chapter, a number of stress conditions can interfere with the function of this organelle and cause abnormal oxidative folding in the ER lumen, resulting in a cellular condition called ER stress. ER stress engages the UPR, an integrated signal transduction pathway that reestablishes homeostasis by increasing the protein folding capacity and quality control mechanisms of the ER. The UPR is activated by three main stresses such as PERK, inositol-requiring transmembrane kinase/endonuclease, and ATF6.

ER and Apoptosis

ER stress activates the UPR, which alleviates this stress by promoting protein folding and clearance to reduce the amount of misfolded proteins at the ER. However, chronic ER stress may result in apoptosis of irreversibly damaged cells through diverse complimentary mechanisms. One such mechanism involves the canonical mitochondrial apoptosis pathway, where the Bcl-2 family plays an important role (Shore et al., 2011). Under irreversible ER stress a switch from the adaptive UPR to pro-apoptotic signaling seems to occur. Although the exact underlying molecular mechanism of this switch is not clear, ER calcium release, Bcl-2 family of proteins, microRNA, and oxidation stress are involved (Urra et al., 2013).

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 the 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 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. Owing to 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.

AGGREPHAGY

The term "aggrephagy" was introduced by Øverbye et al. (2007). Protein aggregation begins with misfolded proteins forming oligomeric intermediates which can mature initially into small protein aggregates, some of which continue to multimerize into aggregates (clumps) of a large size. Both newly formed and preexisting polypeptide chains resulting from genetic mutations, inappropriate protein assembly, aberrant modifications, and environmental stresses are inevitable by-products of biogenesis (Kazami et al., 2011). An aggresome, essentially, is a protein complex containing abnormal proteins, chaperone proteins, proteasome components, mitochondrial components, and ubiquitinated proteins. Proteins of aggresomes are usually ubiquitinated, insoluble, and metabolically stable. Certain other types of aggresome proteins are located at the cell periphery and do not contain ubiquitin (Kaganovich et al., 2008). Protein aggregation in cells is not a simple, random process resulting from uncontrolled interaction among inappropriately exposed hydrophobic surfaces. Some information explaining the formation of protein aggregates is presented below; additional information regarding protein aggregation is found under the heading "Protein Synthesis".

Aggresomes are formed in response to proteasomal inhibition or overexpression of aggregation-prone proteins and are located near the nuclear envelope at the microtubule organizing center. The formation of aggresomes depends on the microtubule-depended transport of protein aggregates (Kopito et al., 2000). Microtubules-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).

Aggregated proteins are toxic, and so their efficient disposal is essential for cell survival. To protect cells from potentially deleterious effects of aggregated proteins, aggresomes are formed to sequester such proteins. Aggresome formation is an active response to cope with excessive levels of misfolded and aggregated proteins. The formation of aggresomes is a second line of active cellular defense. Aggresome formation, in addition, allows the clearance of misfolded proteins by alternative protein quality control systems such as the autophagy–lysosome pathway.

An interesting scheme explaining the formation of protein aggregates in Hela cells and nonsmall lung cancer cells was proposed by Mi et al. (2009). They indicate that cancer chemopreventive ITCs binding to tublin triggers the formation of protein aggregates. This small molecule covalently modifies specific cysteine residues in tubulins, causing transformational changes and misfolding of proteins.

Cells carry out their various functions by synthesizing and degrading proteins on a regular basis. The routine degradation of abnormal (misfolded) and unwanted (excessive) proteins is accomplished primarily by the UPS. The UPS removes nonfunctional, damaged, and/or misfolded proteins from the cell. When the capacity of the proteasome is impaired or when the amount of misfolded proteins exceeds the capacity of proteasome or when the UPS fails to degrade the rapid generation of unfolded proteins, the result is the formation of aggresomes, and the activation of autophagy/lysosome pathway.

The aggregation of misfolded proteins tends to cause problems in the cell. They can damage cells and tissues, and may also cause disorders, including neurodegenerative diseases and type II diabetes. To circumvent such problems and maintain quality control, cells have developed efficient mechanisms to target and eliminate such proteins, which are discussed elsewhere in this chapter. It is difficult to distinguish correctly folded proteins from misfolded proteins of any particular type because both are composed of the same basic amino acid sequence. These two types of proteins differ from each other only in the final three-dimensional folded structures.

Some fundamental questions that still remain to be answered are (Lamark and Johansen, 2012): Which size of protein aggregates are degraded by selective autophagy? Is there a maximum size of the aggregates that can be degraded? Are the large aggregates degraded wholesale or dismantled into small aggregates prior to their engulfment by autophagosomes? What are the differences and similarities between different types of protein aggregates? How should different types of protein aggregates be classified? Is the degradation of large size protein aggregates most efficiently carried out by a combination of UPS, CMA, and autophagy? Can aggrephagy be modulated as a therapeutic strategy for neurodegenerative diseases and other proteinopathies?

AGGRESOME, UBIQUITIN PROTEASOME, AND AUTOPHAGIC SYSTEMS

The UPS removes nonfunctional, damaged, and misfolded proteins from the cell. When the capacity of the proteasome is impaired and/or when the amounts of such proteins exceed the capacity of proteasome, they accumulate into the aggresome. Aggresome formation is a cytoprotective response to sequester potentially toxic misfolded proteins and facilitate their clearance by autophagy, which is related to the UPS and autophagic protein degradation mechanism. Both Parkin and Parkin coregulated gene (*PACRG*) function in aggresome formation and turnover. It has been suggested that this gene functions as a sensor mechanism for the UPS (Taylor et al., 2012). The UPS removes nonfunctional, damaged, and/or misfolded proteins from the cell. When the capacity of the proteasome is impaired and/or when the amount of misfolded proteins exceeds the capacity of proteasome, they accumulate in the form of aggresomes.

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, 2010a,b). 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). They showed that *TRIM50* colocalizes, interacts with, and increases the level of p62 that 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 the 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.

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 punctum 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, 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, which acts as their primary source. 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.

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, which are discussed 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 to 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 *BECN1* 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 defense by the cell 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–Bcl2 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 to undergo 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 autophagy markers in senescent endothelial cells have been found. 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 defense mechanisms of the cell 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, contribute 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 of 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 the 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 life-span 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 that 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-Lerner 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 (Bcl-2 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; Mohapatra *et al*., 2012). Hirsutanol A, a compound from the fungus *Chondrostereum*, inhibits cell proliferation, elevates ROS levels, 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 Bcl-2, 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 Bcl-2-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). Further investigations are required to understand the interplay between these two important cellular processes.

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 immune response by the cell. 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.

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 (Degterev 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 mitofusin 1 and 2, and Opal, which are located in the outer and inner membranes, respectively. Mitochondrial division is mediated by Drpl, which 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 consist 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 AUTOPHAGY

Autophagy is a more selective process than originally anticipated. Autophagy exhibits significant versatility in its selectivity to degrade cell components. This type of autophagy distinguishes cargo to be degraded from its functional counterpart. Some information on the molecular basis of selective autophagy is available. Selective (macro) autophagy includes three critical stages (Okamoto, 2014): (1) signaling from degradation cues induces downstream events specific for a particular target; (2) regulation of important recognized molecules that tag the targets as the disposable cargo; (3) assembly of core autophagy-related proteins to sequester the specific cargo.

A selective autophagy receptor is required to bind specifically to a cargo and dock onto the forming autophagosomes (phagophore), facilitating autophagic sequesteration and degradation of the cargo. Such receptors engage the substrate with the autophagy machinery; examples are Atg32 for mitophagy and Atg19 for the cytoplasm to vacuole targeting pathway. Autophagosomes are specifically generated around the cargo to be degraded via

recognition by autophagy receptors (p62, NBR1). Autophagy receptors/adaptors provide mechanistic insight into selective autophagy process.

Autophagy selectivity is accomplished via the LC3-interacting region (L1R) motif, which ensures the targeting of autophagy receptors to LC3 (or other Atg8 family proteins) anchored in the phagophore membrane (Birgisdottir et al., 2013). Specific autophagy receptors sequester specific cargo into autophagosomes. L1R-containing proteins include cargo receptors, members of the basal autophagy apparatus, proteins associated with vesicles and their transport, Rab/GTPase-associated proteins, and the specific signaling proteins, each of which is degraded by selective autophagy (Birgisdottir et al., 2013). These proteins interact with Atg8 proteins, resulting in the recruitment of cargo to the inner surface of the phagophore and for the recruitment of effector proteins to the outer phagosomal membrane where these effectors mediate transport and maturation of autophagosomes.

The first selective autophagy receptor to be identified was p62 (sequestosome-1 (SQSTM1)) (Bjorkoy et al., 2005). The human p62 protein is 440 amino acids long and contains an N-terminal PB1 domain followed by a zz-type zinc finger domain, nuclear localization signals, unclear export signal, LC3-interacting region, K1R motifs, and a terminal Ub-associated domain (Johansen and Lemark, 2011). p62 harbors active nuclear import and export signals and shuttles between the nucleus and cytoplasm. It is known that p62 acts as a scaffold protein in signaling pathways involving NF-KB and accumulates in ubiquitin-containing protein inclusions in many protein-aggregation diseases (e.g., Alzheimer's disease) (Zatloukal et al., 2002). It is also known that p62 is both a selective autophagy substrate and a cargo receptor for autophagic degradation of ubiquitinated protein aggregates (Bjorkoy et al., 2005). p62, in addition, binds both ubiquitin and LC3 and is removed by autophagy; autophagy blockage results in the failure to degrade p62 that leads to protein aggregation (Komatsu and Ichimura, 2010b).

p62 recruits autophagy adapter ALFY protein (autophagy-linked FYE protein, encoded by the gene located on chromosome 4q21) that in turn recruits the core autophagy machinery ULK1 complex and Vps34 complex (Lin et al., 2013). Both bring the cargo in contact with the core autophagy machinery, allowing the formation of the autophagosomal membrane around the cargo, allowing its sequestration (Isakson et al., 2013). A direct interaction between these adaptors and the autophagosomal marker protein LC3 is required for specific recognition of substrates and efficient selective autophagy (Johansen and Lemark, 2011). (The best described adaptor protein is yeast Atg11 involved in the Ctv pathway.) The cargo consists of ubiquitinated protein aggregates. ALFY is mainly located in the nucleus under normal conditions, but is transferred to the cytoplasm as protein aggregates upon cellular stress. These receptors do not seem to be involved in the bulk degradative autophagy.

NBR1 (neighbor of BRCA1 gene 1) is a protein that is ubiquitously expressed and highly conserved in eukaryotes. This protein is associated with cellular signaling pathways. NBR1 is a binding partner of autophagy-related protein 8 (ATG8) family proteins, including LC3. The ATG8 functions in the formation of autophagosomes, similar to yeast ATG8. NBR1 functions as a cargo adaptor for autophagic degradation of ubiquitinated substrates in a similar way as does p62. Recent studies indicated that NBR1 is located in Lewy bodies and glial cytoplasmic inclusions in multiple system atrophy, suggesting that it has a binding preference for α-synucleinopathy-related molecules (Odagiri et al., 2012).

Relatively recently it was clarified that there are two types of autophagy, starvation-induced autophagy and selective authophagy, each with a different role, although both types use the same autophagy core machinery. Guillebaud and Germain (chapter in this volume) have compared selective autophagy with starvation-induced autophagy and report that these two types are partially distinct from each other; the former degrades large intracellular aggregates and dysfunctional organelles to lessen the occurrence of neurodegenerative diseases, while the latter promotes nutrient recycling and survival. It is suggested that the starvation-induced autophagy may promote survival of cancer cells, while the selective autophagy plays a survival role in neurons by preventing the accumulation of potentially toxic damaged cellular components. Fourteen different types of selective autophagy are 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).

Axonophagy (Neuronal Autophagy)

Selective degradation of axons under pathological conditions is termed axonophagy, which is directly linked to CNS and spinal cord neurodegenerative disorders, including Parkinson's disease, Alzheimer's disease, Huntington's disease, and amyotrophic lateral sclerosis. They exhibit axonal degeneration early in the disease course; examples are degeneration of nigrostriatal projection tracts in Parkinson's disease and corticospinal tracts in amyotrophic lateral sclerosis.

Neurons have developed specific mechanisms for regulating autophagy. However, neuronal autophagic activities can be altered by pathological conditions including neurodegenerative diseases as shown by the accumulation of autophagosomes (Rubinszstein et al., 2005). Large numbers of autophagosomes are frequently found in axonal dystrophic terminals of degenerating neurons (Yue, 2007). Autophagy is more pronounced in axons than in the cell body and dendrites under excitotoxic insult. It has been proposed that p62/SQSTM1 (a putative autophagic substrate) can serve as a marker for evaluating the impairment of autophagic degradation (Yue, 2007). Autophagosomes formed in the distal ends of axons may undergo retrograde axonal transport back to the cell body where lysosomes are usually located for completion of degradation.

Sequential features of axonal degeneration are elaborated below. The distal part of the lesioned axon undergoes initial axonal stability, followed by rapid degeneration and blebbing of the remaining axons, microtubule disassembly, and phagocytic clearance of the lesion site (Knöferle et al., 2010). In contrast to this mechanism, axon degeneration occurs within the first few minutes after lesion, such as in the case of acute axonal degeneration in the spinal cord. One of the putative initiating steps in axonal degeneration is the influx of extracellular calcium, which destabilizes the axon and transmits apoptotic signals to

the neuronal soma (Ziv and Spira, 1995). The role of calcium and autophagosomes in axonophagy is discussed below.

Autophagosomes play a critical role in the axonophagy process, and calcium plays a crucial role in their formation. This process has been investigated in the axonal degeneration in the optic nerve *in vivo* (Knöferle et al., 2010). It was shown that mechanical injury to the optic nerve induced extracellular calcium entry to the axolemma via calcium channels, which resulted in rapid increase of Ca^{2+}. This results in secondary generation of autophagosomes and axonal degradation.

Chromatophagy

It is known that autophagy is the principal catabolic prosurvival pathway during nutritional starvation. However, excess autophagy can be cytotoxic, resulting in cell death. The leakage of DNA, histones, and other chromatin associated proteins are captured by autophagosomes, and this process is referred to as chromatin autophagy or chromatophagy.

Chromatophagy creates an environment within the cell, and as a result, organelles cannot function correctly, resulting in the activation of autophagy. It is known that autophagy via lysosomes degrades dysfunctional organelles. In the absence of functional organelles, cell, of course, will die. It is also known that starvation activates autophagy. For example, when cell is deprived of amino acid arginine (using arginine deiminase), mitochondria become dysfunctional, and produce large amounts of oxidation compounds, which damage the chromatin (proteins and DNA), activating autophagy (Changou et al., 2014). Arginine starvation specifically kills tumor cells by a novel mechanism involving mitochondria dysfunction, generation of reactive oxygen species (ROS), DNA leakage, and chromatin autophagy (chromatophagy). A huge level of giant autophagosomes and autolysosomes encapsulating the leaked DNA is found in the arginine-deprived dying cells (Changou et al., 2014). The specificity mentioned above is expected because tumor cells and normal cells differ in their metabolic requirements. The most prominent examples are addiction of tumor cells to glucose (the Warburg effect) and to glutamine (Vander Heiden et al., 2009; Dang, 2010). Starvation of cells from amino acids, especially from arginine and glutamine, results in the death of cells, preferentially tumor cells.

Argininosuccinate synthetase 1, a rate-limiting enzyme for intracellular arginine synthesis, shows reduced expression in many cancer types such as bladder cancer (Allen et al., 2014). Another example is treatment of prostate cancer cells with ADI-PEG20, which causes DNA leakage, and this DNA together with histones and other chromatin-associated proteins are captured by LC3-containing autophagosomes. Pegylated arginine deiminase (a recombinant mycoplasma protein) converts arginine to citrulline and removes extracellular arginine. This approach has been approved by the FDA for phase III clinical trials in hepatocellular carcinoma (You et al., 2013). Thus, amino acid starvation therapies against tumors have been developed and have reached to clinical trials (Cheng et al., 2007). The starvation therapies have the advantage over radiation and chemotherapy because the former show lower toxicity.

Cell death caused by chromatophagy differs from that by apoptosis. Piecemeal microautophagy of the nucleus (PMN) also differs from chromatophagy because PMN occurs in lower eukaryotic cells, and degradation of nuclear cargo is through nuclear-vacuole

junctions and does not involve the formation of an autotphagosome (Kung et al., 2015). In contrast, chromatophagy cell death is induced by a combination of excessive autophagy and ROS. Chromatophagy is accomplished via a double-membraned structure, derived from fusion between an autophagosome membrane and the nuclear membrane.

As mentioned above, chromatin is leaked from the nucleus because of elevated level of ROS. The concept of the involvement of ROS is strengthened by the evidence that *N*-acetylcysteine (ROS scavenger) reduces chromatophagy phenotype (Kung et al., 2015). Excessive ROS damages nuclear DNA and membrane and induces autophagy, which is observed in chromatophagy. However, it needs to be noted that ROS modulates authophagy and vice versa.

Some information regarding the role of mitochondria in chromatophagy is available. Prolonged arginine depletion impairs mitochondrial oxidative phosphorylation function and depolarizes mitochondrial membrane potential. Thus, ROS production increases significantly in both cytosolic and mitochondrial fractions, leading to the accumulation of DNA damage (Changou et al., 2014). This evidence suggests that mitochondrial damage is central to linking arginine starvation and chromatophagy. This suggestion is supported by knowing that the additions of ROS scavenger *N*-acetylcysteine or knockdown of Atg5 or Beclin 1 reduces chromatophagy.

Arginine starvation has emerged as a potential therapy for cancers as they show selective deficiency of the arginine metabolism. Thus, arginine depletion by the enzyme arginine deiminase induces a cytotoxic autophagy in cancer cells. A novel phenotype with giant autophagosomes, nuclear membrane rupture, and histone-associated DNA leakage encaptured by autophagosomes is found in arginine-deprived dying cells (Changou et al., 2014).

In conclusion, based on the above and other information, mitochondrial ROS regulates chromatophagy (Changou et al., 2014). Chromatophagy has been observed in prostate cancer, pancreatic cancer, and urinary bladder cancer cell lines (Kung et al., 2015). Clinical use of chromatophagy in the future to overcome the resistance of cancer to standard therapies is projected. However, additional information is needed to recommend the ADI-induced chromatophagy for treating malignant diseases. The questions needed to be answered are: How widespread is the phenotype in arginine-depended cancers? Is it restricted to arginine alone, and how can this process be measured *in vivo* (Changou et al., 2014)? For additional related information, see "Nucleophagy" on page 54.

Ciliophagy

Cilia are microtubule-based structures located at the surface of many cell types. An interplay between cilia and autophagy has been reported. Signaling from the cilia recruits the autophagic machinery to trigger autophagosome formation (Orhon et al., 2015). On the other hand, autophagy regulates ciliogenesis by controlling the levels of ciliary proteins.

Sequestration of cilia proteins by autophagy in response to cigarette smoking is termed "ciliophagy." Chronic obstructive pulmonary disease involves aberrant airway inflammatory response to cigarette smoke, which is associated with respiratory epithelial cell cilia shortening and impaired mucociliary clearance (Cloonan et al., 2014). Impaired airway clearance prevents the elimination of dust particles, pathogens, etc., trapped in mucus from the airways.

Increased autophagy in the lungs of chronic obstructive pulmonary disease has been reported (Cloonan et al., 2014). Cilia components function as autophagic substrates during cigarette smoking. Cilia proteins are sequestered within autophagosomes in response to exposure to cigarette smoke. Cilia shortening occurs through an autophagy-dependent mechanism mediated by the histone deacetylase 6, the inhibition of which by tubastatin A, protects mice from cigarette smoking–associated mucociliary dysfunction. Indeed, autophagy-dependent pathway regulates cilia length during cigarette smoking and may disrupt airway epithelial cell function. This disruption reduces epithelial cell cilia length and death of these cells.

Crinophagy

Disposal of excess secretory granules containing insulin by fusion of these granules with lysosomes is termed crinophagy. The β-cells in the pancreatic islets are involved in the storage of insulin secretory granules and instant secretion of insulin. These cells must maintain an optimal insulin concentration, which is maintained by insulin biosynthesis and its intracellular degradation. Such degradation is carried out via crinophagy, that is, β-cell lysosomes are subjected to glucose-dependent alterations. At low or physiological glucose concentration, secretory granules containing insulin are common in β-cell lysosomes.

As mentioned above, crinophagy in these cells is glucose dependent, and variations in glucose concentration affect the balance between insulin biosynthesis and secretion, which is under direct molecular control. It has been demonstrated that intracellular degradation of insulin and crinophagy are regulated by COX-2 activity that is maintained by endogenous nitric oxide (NO) (Sandberg and Borg, 2006). It has also been demonstrated that incubation of isolated pancreatic islets with interleukin 1β (IL-1β) enhances the intracellular degradation of insulin (Sandberg and Borg, 2006). It is known that IL-1β causes expression of inducible nitric oxide synthase (iNOS) in pancreatic islets.

It is concluded that considerable amounts of insulin are degraded within the pancreatic β-cells at low or physiological glucose concentrations, whereas there is virtually no degradation at a high glucose concentration (Halban and Wollheim, 1980). This mechanism seems to control the intracellular degradation of insulin and crinophagy in pancreatic β-cells.

Exophagy

Exophagy is defined as the process by which proteins are secreted into the extracellular space using an unconventional secretion method. The conventional route of passage of proteins is from ribosomes to the ER, Golgi complex, and extracellular space. Signal peptides are involved in this passage. An estimated 30% of human genes encode proteins carrying an N-terminal amino acid sequence that targets most of them to the ER for transportation to the Golgi, and then finally to the extracellular space by the conventional mode of protein secretion.

Some proteins lack an N-terminal signal sequence and do not follow the conventional secretory pathway, and this process is called unconventional protein secretion. Such proteins include insulin-degrading enzymes, angiogenic fibroblast growth factor 1 (FGF1), and interleukin-1X. Several mechanisms have been suggested for the secretion of such proteins (Nickel and Rabouille, 2009), some of which are summarized here.

Acyl coenzyme A (CoA)–binding protein AcbA is one of such proteins, the secretion of which is dependent on Golgi reassembly and stacking protein (GRASP) (Manjithaya et al., 2010). The secretion, processing, and function of an AcbA-derived peptide (SDF-2) are conserved in yeasts *Pichia pastoris* and *Saccharomyces cerevisiae*. It has been shown that in yeast, the secretion of SDF-2-like activity is GRASP-dependent, triggered by nitrogen starvation, and requires autophagy proteins and medium-chain fatty acyl CoA generated by peroxisomes (Manjithaya et al., 2010). Duran et al. (2010) suggest that autophagosomes containing the cargo for unconventional secretion evade fusion with the yeast vacuole, preventing its degradation. These autophagosome intermediates fuse with recycling endosomes and form multivesicular body carriers that then fuse with the plasma membrane to release already-selected cargo. The conserved role of Golgi-associated protein GRASP in starvation-induced unconventional secretion in *Dictyostelium discoideum* has also been reported (Duran et al., 2010). This study indicates the involvement of autophagy genes and the plasma membrane SNARE. Another mechanism explaining the export of such proteins is based on the Cu^{2+}-dependent formation of multiprotein complexes containing the S100A13 protein. Prudovsky et al. (2003) suggest that this protein complex is translocated across the plasma membrane as a "molten globule." This protein is involved in pathological processes. Also, it has been suggested that acyl-coenzyme A-binding protein is sequestered into autophagic vesicles that are subsequently rerouted to the plasma membrane where their content is released into the extracellular space (Abrahamsen and Stenmark, 2010).

Several questions arise. How does the AcbA-containing secretory autophagic vesicle reach the plasma membrane instead of the yeast vacuole? A second question is, what is the difference between AcbA vesicles and degenerative autophagic vesicles? Additionally, how is the cargo sorted for packaging into autophagosomes for extracellular release rather than degradation in lysosomes/vacuoles? It seems that several transport mechanisms are involved in the nonconventional secretion of proteins. Further studies will be required to explicitly clarify the role of autophagosomes/autophagy in the transport of proteins selected for unconventional secretion into the extracellular space.

Glycophagy

The delivery of glycogen to lysosomes for degradation is termed glycophagy. Three types of enzymes convert glucose into uridine 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) and causing 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 a 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 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 Ca^{2+} 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 are 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 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 for damaged mitochondria and marker of 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, enhanced autophagic sequestration of accumulated lipid droplets and damaged mitochondria may occur in the presence of endogenous LC3-II, LAMP-2, PINK1, 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.

Lysophagy

It is known that lysosomes contain acidic hydrolases that degrade cell macromolecules delivered to them via autophagic and endocytic pathways. However, lysosomes can also be ruptured under certain conditions (e.g., pathogenic invasion, bacterial and viral toxins, uptake of minerals), releasing digestive enzymes in the cytosol, which results in the destruction of normal intracellular structures and their functions (Boya and Kroemer, 2008). Lysosomal rupture can also lead to oxidative stress, inflammation, apoptosis, and necrosis. Recently, it was reported that intracellular irritative particles such as human islet amyloid polypeptide, cholesterol crystals, and monosodium urate lead to lysosomal damage (Maejima et al., 2013). As a result, autophagic machinery is recruited only for damaged lysosomes that are engulfed by autophagosomes in mammalian cells (Hung et al., 2013). Such autophagy is indispensable for cellular homeostasis. Lysophagy is thought to be an ubiquitin-mediated process involving LC3 and p62, which contributes to the recovery of lysosomal activities (Maejima et al., 2013). Further information is awaited to explain how ubiquitin and core Atg proteins selectively target damaged lysosomes.

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 the exceedingly complex functions of mitochondria. The major functions of mitochondria are summarized below.

Mitochondria fulfill central roles in oxidative phosphorylation, in 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 non-selective 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, induces mitophagy as well as non-selective autophagy. More importantly, mitochondrial fission is necessary for the induction of mitophagy.

Nucleophagy

Cell nucleus is an organelle bounded by a double membrane, which undergoes drastic reorganization during major cellular events such as cell division and apoptosis. Nucleophagy (macroautophagy) is involved in the elimination of whole nuclei, micronuclei, or chromatin; for additional information related to chromatin elimination, see "Chromatophagy". Alternatively, only parts of the cell nucleus can be selectively degraded without killing the cell. It is known that nucleophagy plays a part in the maintenance of genome stability. Nucleophagy is evoked after genotoxic stress in the context of various biological processes, including endopolyploidy. Failure of DNA repair serves as a signal for the chromatin autophagy of micronuclei. Recently, the elimination of micronuclei from osteosarcoma cells was reported by Rello-Varona et al. (2012). Nucleophagy in multi-nucleated cells favors depolyploidization. This process mitigates aneuploidy with its adverse effects, promoting the survival fitness of decendents and treatment resistance (Erenpreisa et al., 2012). 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 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). These authors employed a genetically encoded nuclear fluorescent reporter (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 – are 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. For additional related information, see Chromatophagy, above.

Pexophagy

The peroxisome organelle is found in humans (especially in liver and kidney), fungi, protozoa, algae, and plants. It is surrounded by a single membrane. Peroxisome numbers are highly regulated in a cell in response to changes in the metabolic status, depending on the cellular needs. They are required for the synthesis of essential cellular components such as plasmogens, isoprenoids, and lysine (Farré et al., 2013). Peroxisomes are dynamic metabolic organelles that are required for oxidation of fatty acids and reduction of hydrogen peroxide produced during lipid oxidation (Deosaran et al., 2013). Peroxisomes also break down methanol, ethanol, formaldehyde, and some types of amino acids. Peroxisomes are also involved in antiviral innate immunity and antiviral signaling (Dixit et al., 2010). The inability to maintain adequate number of peroxisomes is linked to various neurodegenerative diseases.

The selective degradation of dysfunctional peroxisomes by autophagy is referred to as pexophagy. Pexophagy is increased or decreased in response to changes in the metabolic state of the cell or the tissue. The substrate selection is mediated by ubiquitylation recruitment of ubiquitin-binding autophagic receptors, including NBR1, p62, NDP52, and Optineurin. Mutagenesis studies of the NBR1 receptor indicate that the amphipathic α-helical domain, the ubiquitin-associated (UBA) domain, the LC3-interacting region, and coiled-coil domain are necessary to mediate mammalian pexophagy (Deosaran et al., 2013). These authors indicate that although p62 is not essential in the presence of sufficient NBR1, the binding of the former to the latter increases the efficiency of the NBR1-mediated pexophagy. Thus, NBR1 is the specific autophagic receptor for pexophagy in mammalian cells as NBR1 can promote pexophagy in the absence of p62.

Role of Pexophagy in Yeast

The role of pexophagy in the lives of at least three types of yeasts (*Pichia pastoris*, *Saccharomyces cerevisiae*, and *Hansenula polymorpha*) has been extensively studied. Autophagy in these organisms is mainly a survival response to nutrient starvation. Two receptors have been described in *S. cerevisiae*: Atg19 (cytoplasmic-to-vacuole targeting, Cvt) pathway and Atg36, one receptor in *P. pastoris*: Atg30, and two receptors, Pex5p and Pex20p, in *H. polymorpha*. Atg19 interacts directly with the cargo (aminopeptidase 1 (Apel 1)) to form the Cvt complex, and subsequently with two autophagy proteins, Atg11 and Atg8 (Shintani et al., 2002). Atg11 is a required protein for most selective autophagy pathways in yeast and functions as a basic scaffold in assembling the specific phagophore assembly site (PAS) by interacting directly with the receptor, with itself, and several other proteins such as Atg1, Atg9, and Atg17

to form the PAS (Yorimitsu and Klionsky, 2005). Some information is available on how the above-mentioned and other receptors interact with their partners. Farré et al. (2013) report the presence of a phosphoregulatable Atg8-family-interacting motif on Atg30, Atg32, and Atg36; this motif is required for their interactions with Atg8. Mutations of these consensus motifs explain the mechanism of interactions between the receptors and the autophagy proteins.

Atg30 protein is located on the peroxisomal membrane via its interaction with two peroxisomal proteins, Rex14p and Pex3p (Farré et al., 2008). In contrast to mammalian pexophagy receptors, Atg30 does not directly interact with Atg8, but interacts with Atg11 and Atg17 in order to target peroxisomes to autophagosomes. This interaction requires phosphorylation of Atg30. Atg30 receptor, in addition, does not have an ubiquitin-binding domain, indicating that its targeting is not ubiquitin-dependent. However, in *S. cerevisiae*, another receptor, Atg36, binds to Pex3p on peroxisomes and to Atg8 and the adaptor Atg11 (Motley et al., 2012).

The role of the specific protein, PpAtg30, in mediating peroxisome selection during pexophagy of *P. pastoris* has been explained (Farré et al., 2008). Although this protein is required for pexophagy, it is not necessary for other selective or nonselective autophagy-related processes. During pexophagy, this protein is subjected to multiple phosphorylations, at least one of which is required for pexophagy. PpAtg30 is considered to be an important player in selecting peroxisomes as the cargo and its delivery to the autophagic machinery for pexophagy (Farré et al., 2008).

It has been shown in yeast that PpAtg9 is essential for the formation of 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 contrast, during macropexophagy peroxisomes are sequestered primarily by inclusion within the newly formed membranes. Subsequently, the peroxisome-containing pexoautophagosome fuses with the vacuole to deliver its cargo. In the light of the difference in the sequestering mechanism between micropexophagy and macropexophagy, the former process requires a higher level of ATP.

Different types of peroxisome degradation systems have been found in *H. polymorpha* (Manivannan et al., 2013): (1) glucose-induced selective pexophagy serves to degrade peroxisome-containing enzymes that are redundant for growth, and (2) under nitrogen starvation conditions, peroxisomes are degraded by nonselective microautophagy (Bellu and Kiel, 2003). Peroxisome degradation occurs during normal vegetative growth of the yeast cells to continuously rejuvenate this organelle (Aksam et al., 2007).

A recent study by Manivannan et al. (2013) indicates that protein aggregate–containing peroxisomes undergo fission/degradation. They obtained this information by introducing protein aggregates in the organelle matrix. Production of a mutant variant of peroximal catalase results in the formation of large intraorganellar protein aggregates. It is known that protein aggregates are toxic in eukaryotic cells, and their accumulation causes the generation of ROS. The removal of such peroxisomes is an example of quality control process. Pex11 proteins are thought to be actively involved in the recruitment and/or assembly of the peroxisomal fission (Schrader et al., 2012). Asymmetric fission results in the formation

of small and large organelles. According to Manivannan et al. (2013), small organelles are preferentially degraded by pexophagy; however, according to Veenhuis et al. (1983), larger peroxisomes are preferentially degraded. These two studies were carried out under different conditions.

As stated earlier, pexophagy has been extensively studied in the methylotrophic yeast *P. pastoris* that is capable of growth on methanol as a sole source of carbon and energy. Peroxisomes can be rapidly and selectively degraded when methanol-grown yeast cells are placed under 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 yeast cells are placed in an ethanol medium, termed "macropexophagy." In other words, micropexophagy is induced by glucose, while macropexophagy by ethanol. The micro- and macropexophagy pathways are morphologically similar to the micro- and macrophagy pathways, respectively. The introduction of these two pathways depends on the carbon source in the methylotrophic yeast (Ano et al., 2005).

On the other hand, phthalate esters can cause a marked proliferation of peroxisomes. It has been demonstrated in the 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 the 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. During micropexophagy, peroxisomes are incorporated directly into the vacuole by invagination.

Finally, it can be deduced from the above discussion that the process of pexophagy in the Cvt pathway with regard to its turnover, autophagic adaptor proteins, and peroxisome engulfment is more complex than that described above. For example, Grunau et al. (2011) have discovered the PtdIas3P-synthesizing activity in peroxisomes of *S. cerevisiae*, and lipid kinase Vps34p is associated with peroxisomes during their biogenesis. Although Vps34p is involved in such biosynthesis, this kinase is not essential for optimal peroxisome biogenesis. It seems that Vps34p-containing complex I and a subset of PtdInos3p-binding proteins are required for the regulated degradation of peroxisomes.

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 takes 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).

Both autophagy-dependent and autophagy-independent systems are involved in reticulophagy. Accumulation of excess membrane proteins on the aberrant ER induces ER stress and blocks the transportation of these membranes to the lysosome. Recently, it was reported that the conserved Ypt/Rab GTPases regulate reticulophagy (Lipatova et al., 2013). A Ypt/Rab GTPase module consisting of the Tris85-containing TRAPP111 GEF, Yptl, and the Atgll effector regulates shuttling of tagged, overexpressed, and misfolded proteins from the ER to the autophagic pathway (Lipatova et al., 2013). It is known that these GTPases regulate trafficking between cellular components. These proteins are stimulated by guanine-nucleotide exchange factors (GEFs) to recruit multiple effectors, which mediate all vesicular transport events (Segev, 2001). It is also known that Ypt regulates ER-to-Golgi transport and autophagosome formation (Chua et al., 2011). Indeed, Ypt has multiple roles.

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 fusion, 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.

The unfolded protein response (UPR) is a form of intracellular signaling triggered by 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. The 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-Hansen 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) ER-associated degradation (ERAD) (Bernales et al., 2006a; 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. The UPR also initiates inhibition of general translation and upregulation of genes encoding ER chaperones and components of ERAD machinery (Cebollero et al., 2012). 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).

In the absence of or inefficient reticulophagy, misfolded or unfolded proteins accumulate on the ER membranes; examples of such proteins are: α-synuclein (Parkinson's

disease), amyloid protein (Alzheimer's disease), htt protein (Huntington's disease), FUS protein (amyotrophic lateral sclerosis), and PrP (prion disease). These accumulated proteins on the ER membrane are implicated in neurodegenerative diseases. Under such circumstances, cells fail to maintain protein homeostasis (proteostasis) and elicit unfolded protein response (UPR). UPR serves to attenuate protein translation and increase protein refolding or degradation.

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 (Macintosh and Bassham, 2011). 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 (Macintosh and Bassham, 2011).

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 (Beau et al., 2008).

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 (Baxter et al., 2005).

It is concluded that ribosome degradation relies on both ribophagy and non-selective autophagy. The evidence presented here 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 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 pancreatitis. In fact, a VMP1-USP4-p62 molecular pathway is involved in mitophagy.

As explained above, if zymogen granules prematurely release digestive enzymes in 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.

References

Abrahamsen, H., and Stenmark, H., 2010. Protein secretion: unconventional exit by exophagy. Curr. Biol. 20, 415–418.

Agarraberes, F.A., Dice, J.F., 2001. A molecular chaperon complex at the lysosomal membrane is required for protein translocation. J. Cell Biol. 114, 2491–2499.

Ajabnoor, G.M.A., Crook, T., Coley, H.M., 2012. Paclitaxel resistance is associated with switch from apoptotic to autophagic cell death in MCF-7 breast cancer cells. Cell Death Dis. 3, 1–9.

Akira, S., Uematsu, S., Takeuchi, O., 2006. Pathogen recognition and innate immunity. Cell 124, 783–801.

Aksam, E.B., Koek, A., Kiel, J.A.K.W., et al., 2007. A peroxisomal ion protease and peroxisome degradation by autophagy play key roles in vitality of *Hansenula polymorpha* cells. Autophagy 3, 96–105.

Al Rawi, S., Louvet-Vallee, S., Djeddi, A., et al., 2012. Allophagy: a macroautophagic process degrading spermatozoid-inherited organelles. Autophagy 8, 421–423.

Allen, M.D., Luong, P., Hudson, C., et al., 2014. Prognostic and therapeutic impact of argininosuccinate synthetase 1 control in bladder cancer as monitored longitudinally by PET imaging. Cancer Res. 74, 896–907.

Ano, Y., Hattori, T., Kato, N., et al., 2005. Intracellular ATP correlates with mode of pexophagy in *Pichia pastoris*. Biosci. Biotechnol. Biochem. 69, 1527–1533.

Andrade, D.M., Turnball, J., Minassian, B.A., 2007. Lafora disease, seizures and sugars. Acta Myol. 26, 83–86.

Aoki, H., Kondo, Y., Aldape, K., et al., 2008. Monitoring autophagy in glioblastoma with antibody against isoform B of human microtubule-associated protein 1 light chain 3. Autophagy 4, 467–475.

Austin, R.H., Beeson, K.W., Eisenstein, L., et al., 1975. Dynamics of ligand-binding to myoglobin. Biochemistry 14, 5355–5373.

Ave, E.L., Walker, S.A., Manifava, M., et al., 2008. Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. J. Cell Biol. 182, 685–701.

Bakowska-Zywicka, K., Tyczewska, A., Twardowski, T., 2006. Mechanism of peptide bond formation on the ribosome – controversions. In Polish. Postepy Biochem. 52, 166–172.

Baxter, B.K., Abeliovich, H., Zhang, X., et al., 2005. Atg19p ubiquitination and the cytoplasm to vacuole trafficking pathway in yeast. J. Biol. Chem. 280, 39067–39076.

Beau, I., Esclatine, A., Codogno, P., 2008. Lost to translation: when autophagy targets mature ribosomes. Trends Cell Biol. 18, 311–314.

Bellu, A.R., Kiel, J.A.K.W., 2003. Selective degradation of peroxisomes in yeasts. Miscros. Res. Tech. 61, 161–170.

Bernales, S., McDonald, K.L., Walter, P., 2006a. Autophagy counterbalances endoplasmic reticulum expansion during the unfolded protein response. PLoS Biol. 4 (12), 2311–2324.

Bernales, S., Papa, F.R., Walter, P., 2006b. Intracellular signaling by the unfolded protein response. Annu. Rev. Cell Develop. Biol. 22, 487–508.

Berrym, S., Brooks, E., Burman, A., et al., 2012. Foot-and-mouth disease virus induces autophagosomes during cell entry via a Class III phosphatidylinositol 3-kinase-independent pathway. J. Virol. 86, 12940–12953.

Birgisdottir, A.B., Lamark, T., Johansen, T., 2013. The LIR motif—crucial for selective autophagy. J. Cell Sci. 126, 3237–3247.

Bjorkoy, G., Lamark, T., Brech, A., et al., 2005. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on Huntington-induced cell death. J. Cell Biol. 171, 603–614.

Blanchet, F.P., Morris, A., Nikolic, D.S., et al., 2010. Human immunodeficiency virus-1 inhibition of immunoamphisomes in dendritic cells impairs early innate and adaptive immune responses. Immunity 32, 654–669.

Borgia, A., Williams, P.M., Clarke, J., 2008. Single-molecule studies of protein folding. Annu. Rev. Biochem. 77, 101–125.
Boya, P., and Kroemer, G., 2008. Lysosomal membrane permeabilization in cell death. Oncogene 27, 6434–6451.
Braakman, I., Bulleid, N.J., 2011. Protein folding and modification in the mammalia endoplasmic veticulum. Annu. Rev. Biochem. 80, 71–99.
Bringer, M.-A., Lapaquette, P., Nguyen, H., et al., 2014. Polymorphisms in autophagy-related genes in Crohn's disease: impact on intracellular bacteria persistence and inflammatory response. In: Hayat, M.A. (Ed.), Autophagy: Cancer, Other Pathologies, Immunity, Infection, and Aging Academic Press, New York, NY, pp. 95.
Brodsky, F.M., 1988. Living with clathrin: its role in intracellular membrane traffic. Science 242, 1396–1402.
Cadwell, K., Patel, K.K., Komatsu, M., et al., 2009. A common role for Atg16L1, Atg5, and Atg7 in small intestinal Paneth cells and Crohn disease. Autophagy 5, 250–252.
Cebollero, E., Reggiori, F., Kraft, C., 2012. Reticulophagy and ribophagy: regulated degradation of protein production factories. Int. J. Cell Biol. 2012, 182834.
Cemma, M., Brumell, J.H., 2012. Interactions of pathogenic bacteria with autophagy systems. Cur. Biol. 22, 540–545.
Chakrabarti, A., Gosh, P.K., Banerjee, S., et al., 2012. RNase L triggers autophagy in response to viral infections. J. Virol. 86, 11311–11321.
Chang, T., Schroder, L.A., Thomson, J.M., et al., 2005. PpAtg9 encodes a novel membrane protein that traffics to vacuolar membranes, which sequester peroxisomes during pexophagy in *Pichia pastoris*. Mol. Biol. Cell 16, 4941–4953.
Changou, C.A., Chen, Y.-R., Li, X., et al., 2014. Arginine starvation-associated atypical cellular death involves mitochondrial dysfunction, nuclear DNA leakage, and chromatin autophagy. Proc. Natl. Acad. Sci. U. S. A. 111, 14147–14152.
Chen, G., Hu, X., Zhang, W., et al., 2012. Mammalian target of rapamycin regulates isoliquiritigenin-induced autophagic and apoptotic cell death in adenoid cystic carcinoma cells. Apoptosis 17, 90–101.
Chen, Y., Yu, L., 2012. Autophagic lysosome reformation. Exp. Cell Res. 319, 142–146.
Chen, Y.-T., Burchell, A., 1995. Glycogen storage diseases. In: Scriver, C.R., Beaudet, A.L., Sly, W.S. (Eds.), The Metabolic and Molecular Basis of Inherited Disease McGraw-Hill, New York, pp. 935–965.
Cheng, P.N., Lam, T.L., Tsui, S.M., et al., 2007. Pegylated recombinant human arginase (rhArg-peg 5,000 mw) inhibits the *in vitro* and *in vivo* proliferation of human hepatocellular carcinoma through arginine depletion. Cancer Res. 67, 309–317.
Chin, I.S., Olzmann, J.A., Li, L., 2010. Parkin-mediated ubiquitin signaling in aggresome formation and autophagy. Biochem. Soc. Trans. 38, 144–149.
Christian, P., Sacco, J., Adeli, K., 2013. Autophagy: emerging roles in lipid homeostasis and metabolic control. Biochim. Biophys. Acta Mol. Cell Biol. Lipids 1831, 819–824.
Chua, C.E., Gan, B.Q., Tang, B.L., 2011. Involvement of members of the Rab family and related small GTPases in autophagosomes formation and maturation. Cell. Mol. Life Sci. 68, 3349–3358.
Cloonan, S.M., Lam, H.C., Ryter, S.W., et al., 2014. Ciliophagy: the consumption of cilia components by autophagy. Autophagy 10 (3), 532–534.
Cooney, R., Baker, J., Brain, O., et al., 2010. NOD2 stimulation induces autophagy in dendritic cells influences bacterial handling and antigen presentation. Nat. Med. 16 (1), 90–97.
Cortes, C.J., Qin, K., Norstrom, E.M., et al., 2013. Early delivery of misfolded Prp from ER to lysosomes by autophagy. Int. J. Cell Biol. 2013, 18. Article ID 560421.
Cuervo, A.M., 2009. Chaperone-mediated autophagy: selectivity pays off. Trends Endocrinol. Metab. 21, 142–150.
Cuervo, A.M., Dice, J.F., 1996. A receptor for the selective uptake and degradation of proteins by lysosomes. Science 273, 501–503.
Czaja, M.J., 2010. Autophagy in health and disease. 2. Regulation of lipid metabolism and storage by autophagy: pathophysiological implications. Am. J. Physiol. Cell Physiol. 298, C973–C978.
Da Cruz, S., Cleveland, D., 2011. Understanding the role of TDP-43 and FUS/TLS in ALS and beyond. Curr. Opin. Neurobiol. 21, 904–919.
Dang, C.V., 2010. Rethinking the Warburg effect with Myc micromanaging glutamine metabolism. Cancer Res. 70, 859–862.
Das, D.K., 2011. Autophagy: a study of live or let die. Antioxid. Redox Signal. 14, 2163–2164.
Deegan, S., Saveljeva, S., Gorman, A.M., et al., 2013. Stress-induced self-cannibalism: on the regulation of autophagy by endoplasmic reticulum stress. Cell Mol. Life Sci. 70, 2425–2441.

Degterev, A., Hitomi, J., Germscheid, M., et al., 2008. Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat. Chem. Biol. 4, 313–321.
Deosaran, E., Larsen, K.B., Hua, R., et al., 2013. NBR1 acts as an autophagy receptor for peroxisomes. J. Cell Sci. 126, 939–952.
DeSelm, C.J., Miller, B.C., Zou, W., et al., 2011. Autophagy proteins regulate the secretory component of osteoclastic bone resorption. Dev. Cell 21, 966–974.
Deter, R.L., de Duve, C., 1967. Influence of glucagon, and inducer autophagy, on some physical properties of rat liver lysosomes. J. Cell Biol. 33, 437–499.
Dice, J., 1990. Peptide sequences that target cytosolic proteins for lysosomal proteolysis. Trends Biochem. Sci. 15, 305–309.
DiFiglia, M., Sapp, E., Chase, K.O., et al., 1997. Aggregation of Huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. Science 277, 1990–1993.
Ding, F., Yin, Z., Wang, H.R., 2011. Ubiquitination in Rho signaling. Curr. Top. Med. Chem. 11, 2879–2887.
Dixit, E., Boulant, S., Zhang, Y., et al., 2010. Peroxisomes are signaling platforms for antiviral innate immunity. Cell 141, 668–681.
Doyle, K.M., Kennedy, D., Gorman, A.M., et al., 2011. Unfolded proteins and endoplasmic reticulum stress in neurodegenerative disorders. J. Cell. Mol. Med. 15, 2025–2039.
Dunker, A., Oldfield, C., Meng, J., et al., 2008. The unfoldomics decade: an update on intrinsically disordered proteins. BMC Genomics 9, S1.
Dupont, N., Temime-Smaali, N., Lafont, F., 2010. How ubiquitination and autophagy participate in the regulation of the response to bacterial infection. Cell 102, 621–634.
Duran, J.M., Anjard, C., Stefan, C., et al., 2010. Unconventional secretion of Acb1 is mediated by autophagosomes. J. Cell Biol. 188, 527–536.
Dyson, H.J., Wright, P.E., 1996. Insights into protein folding from NMR. Annu. Rev. Phys. Chem. 47, 369–395.
Dyson, H.J., Wright, P.E., 2004. Unfolded proteins and protein folding studied by NMR. Chem. Rev. 104, 3607–3622.
Dyson, H.J., Wright, P.E., 2005. Intrinsically unstructured proteins and their functions. Nat. Rev. Mol. Cell Biol. 6, 197–208.
Eid, N., Ito, Y., Maemura, K., et al., 2013. Elevated autophagic sequestration of mitochondria and lipid droplets in steatotic hepatocytes of chronic ethanol-treated rats: an immunohistochemical and electron microscopic study. J. Mol. Histol. 44, 311–326.
Eisenberg-Lerner, A., Bialik, S., Simon, H.-U., et al., 2009. Life and death partners: apoptosis, autophagy, and the cross-talk between them. Cell Death Differ. 16, 966–975.
Erenpreisa, J., Huna, A., Salmina, K., et al., 2012. Macroautophagy-aided elimination of chromatin: sorting of waste, sorting of fate? Autophagy 8, 1877–1881.
Farré, J.C., Manjithaya, R., Mathewson, R.D., et al., 2008. PpAtg30 tags peroxisomes for turnover by selective autophagy. Dev. Cell 14, 365–376.
Farré, J.C., Burkenroad, A., Burnett, S., et al., 2013. Phosphorylation of mitophagy and pexophagy receptors coordinates their interaction with Atg8 and Atg11. EMBO Rep. 14, 441–449.
Ferreon, A.C.M., Moosa, M.M., Gambin, Y., et al., 2012. Counteracting chemical chaperone effects on the single-molecule α-synuclein structural landscape. Proc. Natl. Acad. Sci. U. S. A. 109, 17826–17831.
Florey, O., Overholtzer, M., 2012. Autophagy proteins in macroendocytic engulfment. Trends Cell Biol. 22, 376–380.
Franchi, L., Eigenbrod, T., Munoz-Planillo, R., et al., 2009. The inflammasome: a caspase-1 activation platform regulating immune responses and disease pathogenesis. Nat. Immunol. 10, 241–255.
Fritz, T., Niederreiter, L., Adolph, T., et al., 2013. Crohn's disease: NOD2, autophagy and ER stress converge. Gut 60, 1580–1588.
Fujimoto, T., Ohsaki, Y., Cheng, J., et al., 2008. Lipid droplets: a classic organelle with new outfits. Histochem. Cell Biol. 130, 263–279.
Fujita, E., Kouroku, Y., Isoai, A., et al., 2007. Two endoplasmic reticulum-associated degradation (DRAD) systems for the novel variant of the mutant dysferlin: ubiquitin/proteasome ERAD (11). Hum. Mol. Genet. 16, 618–629.
Fujita, N., Itoh, T., Omori, H., et al., 2008. The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy. Mol. Biol. Cell 19, 2092–2100.
Fukuda, T., Roberts, A., Ahearn, M., et al., 2006. Autophagy and lysosomes in Pompe disease. Autophagy 2, 318–320.
Funderburk, S.F., Wang, Q.J., Yue, Z., 2010. The Beclin 1–VPS34 complex at the crossroads of autophagy and beyond. Trends Cell Biol. 20, 355–362.

Fusco, C., Micale, L., Egorov, M., et al., 2012. The E3-ubiquitin ligase TRIM50 interacts with HDAC6 and promotes the sequesteration and clearance of ubiquitinated proteins into the aggresome. PLos One 7 (issue), e40440.
Gandesiri, M., Chakilam, S., Ivanovska, J., et al., 2012. Dapk plays an important role in panobinostat-induced autophagy and commits cells to apoptosis under autophagy deficient conditions. Apoptosis 17, 1300–1315.
Gao, W., Kang, J.H., Liao, Y., et al., 2010. Biochemical isolation and characterization of tubulovesicular LC3-positive autophagosomal compartment. J. Biol. Chem. 285, 1371–1383.
Gewirtz, D.A., 2014. Autophagy and senescence in cancer therapy. J. Cell. Physiol. 229, 6–9.
Goehe, R.W., Di, X., Sharma, K., et al., 2012. The autophagy-senescence connection in chemotherapy: most tumor cells (self) eat before they die. J. Pharmacol. Exp. Ther. 343, 763–778.
Gorman, A.M., Healy, S.J., Jager, R., et al., 2012. Stress management at the ER: regulators of ER stress-induced apoptosis. Pharmacol. Ther..http://dx.doi.org/10.1016/j.pharmthera.2012.02.003
Gosselin, K., Deruy, E., Martien, S., et al., 2009. Senescent keratinocytes die by autophagic programmed cell death. Am. J. Pathol. 174, 423–435.
Gottlieb, R.A., Mentzer Jr., R.M., 2013. Autophagy: an affair of the heart. Heart Fail. Rev. 18, 575–584.
Grasso, D., Ropolo, A., Lo, Re, A., et al., 2011. Zymophagy, a novel selective autophagy pathway mediated by VMP1-USP9x-p 62, prevents pancreatic cell death. J. Biol. Chem. 286, 8308–8324.
Grunau, S., Lay, D., Mindthoff, S., et al., 2011. The phosphoinositide 3-kinase Vps34p is required for pexophagy in *Saccharomyces cerevisiae*. Biochem. J. 434, 161–170.
Gui, Y.-X., Fan, X.-N., Wang, H.-M., et al., 2012. Glyphosate induced cell death through apoptotic mechanisms. Neurotoxicol. Teratol. 34, 344–349.
Guo, R., Ren, J., 2012. Deficiency in AMK attenuates ethanol-induced cardiac contractile dysfunction through inhibition of autophagosome formation. Cardiovasc. Res. 94, 480–491.
Gyllenstein, U., Wharton, D., Joseffson, A., et al., 1991. Paternal inheritance of mitochondrial DNA in mice. Nature 352, 255–257.
Halban, P.A., Wollheim, C.B., 1980. Intracellular degradation of insulin stores by rat pancreatic islets *in vitro*: an alternative pathway for homeostases of pancreatic insulin content. J. Biol. Chem. 255, 6003–6006.
Hähnel, P.S., Thaler, S., Antunes, E., et al., 2008. Targeting AKT signaling sensitizes cancer to cellular immunotherapy. Cancer Res. 68 (10), 3899–3906.
Harashima, N., Inao, T., Imamura, R., et al., 2012. Roles of PI3K/Akt pathway and autophagy in TLR3 signaling-induced apoptosis and growth arrest of human prostate cancer. Cancer Immunol. Immunother. 61, 667–676.
Hartl, F.U., Hayer-Hartl, M., 2009. Converging concepts of protein folding *in vitro* and *in vivo*. Nat. Struct. Mol. Biol. 16, 574–581.
He, W., Liu, Q., Wang, L., et al., 2007. TLR4 signaling promotes immune escape of human lung cancer cells by inducing immunosuppressive cytokines and apoptosis resistance. Mol. Immunol. 44, 2850–2859.
Hocking, L.J., Whitehouse, A., Helfrich, M.H., 2012. Autophagy: a new player in skeletal maintenance? J. Bone Miner. Res. 27, 1439–1447.
Hoffman, W.H., Shacka, J.J., Andjelkovic, A.V., 2012. Autophagy in the brains of young patients with poorly controlled TIDM and fatal diabetic ketoacidosis. Exp. Mol. Pathol. 93, 273–280.
Hossain, A., Radwan, F.F.Y., Doonan, B.P., et al., 2012. A possible crosstalk between autophagy and apoptosis in generating an immune response in melanoma. Apoptosis 17, 1066–1078.
Hoyer-Hansen, M., Jaattela, M., 2007. Connecting endoplasmic reticulum stress to autophagy by unfolded protein response and calcium. Cell Death Differ. 14, 193–205.
Hu, S.-Y., Tai, C.C., Li, Y.H., et al., 2012. Progranulin compensates for blocked IGF-1 signaling to promote myotube hypertrophy in C2C12 myoblast via the PI3K/Akt/mTOR pathway. FEBS Lett. 586 (19), 3485–3492.
Huang, C., Andres, A.M., Ratliff, E.P., et al., 2011. Preconditioning involves selective mitophagy mediated by Parkin and p26/SQSTM1. PLos ONE 6, 20975.
Hung, Y.H., Chen, L.M., Yang, J.Y., et al., 2013. Spatiotemporally controlled induction of autophagy-mediated lysosome turnover. Nat. Commun. 4, 2111.
Isakson, P., Holland, P., Simonsen, A., 2013. The role of ALFY in selective autophagy. Cell Death Differ. 20, 12–20.
Ishibashi, K., Uemura, T., Waguri, S., et al., 2012. Atg16L1, an essential factor for canonical autophagy, participates in hormone secretion from PC12 cells independently of autophagic activity. Mol. Cell. Biol. 23, 3193–3202.
Jackson, W.T., Giddings Jr., T.H., Taylor, M.P., et al., 2005. Subversion of cellular autophagosomal machinery by RNA viruses. PLos Biol. 3, 861–871.

Janecek, S., 2002. A motif of a microbial starch-binding domain found in human genethonin. Bioinformatics 18, 1534–1537.
Janovick, J., Stewart, D.M., Jacob, D., et al., 2013. Restoration of testis function in hypogonadotropic hypogonadal mice harboring a misfolded GnrHR mutant by pharmacoperones drug therapy. Proc. Natl. Acad. Sci. U. S. A. 110, 21030–21035.
Jiang, S., Wells, C.D., Roach, P.J., 2011. Starch-binding domain-containing protein 1 (Stbd1) and glycogen metabolism: identification of the Atg8 family interacting motif (AIM) in Stbd1 required for interaction with GABARAPL1. Biol. Chem. Res. Commun. 413, 420–425.
Jia, H., Kast, R.J., Steffan, J.S., et al., 2012. Selective histone deacetylase (HDAC) inhibition imparts beneficial effects in Huntington's disease mice: implications for the ubiquitin-proteasomal and autophagy systems. Hum. Mol. Genet. 21, 5280–5293.
Johansen, T., Lemark, T., 2011. Selective autophagy mediated by autophagic adaptor proteins. Autophagy 7 (3), 279–296.
Jung, C.H., Ro, S.H., Cao, J., et al., 2010. mTOR regulation of autophagy. FEBS Lett. 585, 1287–1295.
Kabi, A., McDonald, C., 2014. Protein phosphatase 2A has positive and negative roles in autophagy. In: Hayat, M.A. (Ed.), Autophagy: Cancer, Other Pathologies, Immunity, Infection and Aging Academic Press, New York, NY, pp. 218.
Kadowaki, M., Karim, M.R., 2009. Cytosolic LC3 ratio as a quantitative index of macroautophagy. Methods Enzymol. 452, 199–213.
Kaganovich, D., Kopito, R., Frydman, J., 2008. Misfolded proteins partition between two distinct quality control compartments. Nature 454, 1088–1095.
Kageyama, Y., Zhang, Z., Roda, R., et al., 2012. Mitochondrial division ensures the survival of postmitotic neurons by suppressing oxidative damage. J. Cell Biol. 197, 535–551.
Kageyama, Y., Zhang, Z., Sesaki, H., 2011. Mitochondrial division: molecular machinery and physiological functions. Curr. Opin. Cell Biol. 23, 427–434.
Kang, M.R., Kim, M.S., Oh, J.E., et al., 2009. ATG2B, ATG5, ATG9B, and ATG12 in gastric and colorectal cancers with microsatellite instability. J. Pathol. 217, 702–706.
Kapuy, O., Vinod, P.K., Mandl, J., et al., 2013. A cellular stress-directed bistable switch controls the crosstalk between autophagy and apoptosis. Mol. BioSyst. 9, 296–306.
Karim, R., Kanazawa, T., Daigaku, Y., et al., 2007. Cytosolic LC3 ratio as a sensitive index of macroautophagy in isolated rat hepatocytes and H4-11-E cells. Autophagy 3, 553–560.
Kaushik, S., Rodriguez-Navarro, J.A., Arias, E., et al., 2011. Autophagy in hypothalamic AgRP neurons regulates food intake and energy balance. Cell Metab. 14, 173–183.
Kawai, T., Akira, S., 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat. Immunol. 11, 373–384.
Kawaguchi, Y., Kovacs, J.J., McLaurin, A., et al., 2003. The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. Cell 115, 727–788.
Kazami, S., Usui, T., Osada, H., 2011. Actin stress fiber retraction and aggresome formation is a common cellular response to actin toxins. Biosci. Biotechnol. Biochem. 75, 1853–1855.
Kidgotko, O.V., Kustova, M.Y., Sokolova, V.A., et al., 2013. Transmission of human mitochondrial DNA along the paternal lineage in transmitochondrial mice. Mitochondrion 13, 330–335.
Killian, M.S., 2012. Dual role of autophagy in HIV-1 replication and pathogenesis. AIDS Res. Ther. 9, 16.
Kim, H.-S., Montana, V., Jang, H.J., et al., 2013. Epigallocatechin-gallate (EGCG) stimulates autophagy in vascular endothelial cells: a potential role for reducing lipid accumulation. J. Biol. Chem. 288, 22693–22705.
Kimura, S., Fujita, N., Noda, T., et al., 2009. Monitoring autophagy in mammalian cultured cells through the dynamics of LC3. Methods Enzymol. 452, 1–12.
Kirkin, V., McEwan, D., Novak, I., et al., 2009. A role for ubiquitin in selective autophagy. Mol. Cell 34, 259–269.
Klionsky, D.J., Abdalla, F.C., Abeliovich, H., et al., 2012. Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy 8, 445–544.
Knaevelsrud, H., Ahlquist, T., Merok, M.A., et al., 2010. UVRAG mutations associated with microsatellite unstable colon cancer do not affect autophagy. Autophagy 6, 863–870.
Knöferle, J., Koch, J.C., Ostendorf, T., et al., 2010. Mechanisms of acute axonal degeneration in the optic nerve *in vivo*. PNAS 107, 6064–6069.

Komatsu, M., Ichimura, Y., 2010a. Selective autophagy regulates various cellular functions. Genes Cells 15, 923–2010.
Komatsu, M., Ichimura, Y., 2010b. Physiological significance of selective degradation of p62 by autophagy. FEBS Lett. 584, 1374–1378.
Kopito, R.R., 2000. Aggresomes, inclusion bodies and protein aggregation. Trends Cell Biol. 10, 524–530.
Kraft, C., Deplazes, A., Sohrman, M., et al., 2008. Mature ribosomes are selectively degraded upon starvation by an autophagy pathway requiring the Ubp3p/Bre5p ubiquitin protease. Nat. Cell Biol. 10, 602–610.
Kroemer, G., Marino, G., Levine, B., 2010. Autophagy and the integrated stress response. Mol. Cell 40, 280–293.
Kuma, A., Matsui, M., Mizushima, N., 2007. LC3, and autophagosome marker, can be incorporated into protein aggregates independent of autophagy. Autophagy 3, 323–328.
Kung, H.-J., Changou, C.A., Li, C.-F., et al., 2015. Chromatophagy: autophagy goes nuclear and captures broken chromatin arginine-starvation. Autophagy 11 (2), 419–421.
Kvam, E., Goldfarb, D.S., 2004. Nvj1p is the outer-nuclear-membrane receptor for oxysterol-binding protein homolog Osh1p in *Saccharomyces cerevisiae*. J. Cell Sci. 117, 4959–4968.
Kyei, G.B., Dinkins, C., Davis, A.S., et al., 2009. Autophagy pathway intersects with HIV-1 biosynthesis and regulates viral yields in macrophages. J. Cell Biol. 186, 255–268.
Lamark, T., Johansen, T., 2012. Aggrephagy: selective disposal of protein aggregates by macroautophagy. Int. J. Cell Biol., 21. http://dx.doi.org/10.1155/2012/736905.
Lee, A.C., Fenster, B.E., Ito, H., et al., 1999. Ras proteins induce senescence by altering the intracellular levels of reactive species. J. Biol. Chem. 274, 7936–7940.
Li, Y., Hu, Z., Chen, B., et al., 2012. Taurine attenuates methamphetamine-induced autophagy and apoptosis in PC12 cells through mTOR signaling pathway. Toxicol. Lett. 215, 1–7.
Liang, X.H., Jackson, S., Seaman, M., et al., 1999. Induction of autophagy and inhibition of tumorigenesis by Beclin 1. Nature 402, 672–676.
Lin, X., Li, S., Zhao, Y., et al., 2013. Interaction domains of p62: a bridge between p62 and selective autophagy. DNA Cell Biol. 32, 220–227.
Lipatova, Z., Shah, A.H., Kim, J.J., et al., 2013. Regulation of ER-phagy by a Ypt/Rab GTPase module. Mol. Biol. Cell 24, 3133–3143.
Liu, Y., Tan, Y.L., Zhang, X., et al., 2014. Small molecular probes to quantify the functional fraction of a specific protein in a cell with minimal folding equilibrium shifts. Proc. Natl. Acad. Sci. U. S. A. 111, 4449–4454.
Liu, E.Y., Ryan, K.M., 2012. Autophagy and cancer: issues we need to digest. J. Cell Sci. 125, 2349–2358.
Lo Ré, A.E., Fernández-Barrena, M.G., Almada, L.L., et al., 2012. Novel AKT1–GL13–VWP1 pathway mediates KRAS oncogene-induced autophagy in cancer cells. J. Biol. Chem. 287, 25325–25334.
Lozy, F., Karantza, V., 2012. Autophagy and cancer cell metabolism. Rev. Cell Dev. Biol. 23, 395–401.
Lu, J.V., Walsh, C.M., 2012. Programmed necrosis and autophagy in immune function. Immunol. Rev. 249, 205–217.
Luo, S., Rubinsztein, D.C., 2010. Apoptosis blocks Beclin 1-dependent autophagosome synthesis: an effect rescued by Bcl-xL. Cell Death Differ. 17 (268), 277.
Macintosh, G.C., Bassham, D.C., 2011. The connection between ribophagy and ribosomal RNA decay. Autophagy 7 (6), 662–663.
Maejima, I.A., Takahashi, H., Omori, T., et al., 2013. Autophagy sequesters damaged lysosomes to control lysosomal biogenesis and kidney injury. EMBO J. 32, 2336–2347.
Manivannan, S., de Boer, R., Vennhuis, M., et al., 2013. Lumenal peroxisomal protein aggregates are removed by concerted fission and autophagy events. Autophagy 9 (7), 1044–1056.
Manjithaya, R., Anjard, C., Loomis, W.F., et al., 2010. Unconventional secretion of Pichia pastoris Acb1 is dependent upon GRASP protein, peroxisomal functions, and autophagosome formation. J. Cell Biol. 188, 188–546.
Master, S., Rampini, S., Davis, A., et al., 2008. *Mycobacterium tuberculosis* prevents inflammasome activation. Cell Host Microbe 3, 224–232.
McLaughlin, M., Vandenbroeck, K., 2011. The endoplasmic reticulum protein folding factor and its chaperones: new targets for drug discovery. Br. J. Pharmacol. 162, 328–345.
Mei, Y., Su, M., Soni, G., et al., 2014. Intrinsically disordered regions in autophagy proteins. Proteins 82, 565–578.
Meijer, A.J., Godogno, P., 2004. Regulation and role of autophagy in mammalian cells. Int. J. Biochem. Cell Biol. 36, 2445–2462.
Mercer, T.R., Neph, S., Dinger, M.E., et al., 2011. The human mitochondrial transcriptome. Cell 146, 645–658.

Mi, L., Gan, N., Chung, F.-L., 2009. Aggresome-like structure induced by isothiocyanates is novel proteasome-dependent degradation machinery. Biochem. Biophys. Res. Commun. 388, 456–462.
Mijaljica, D., Devenish, R.J., 2013. Nucleophagy at a glance. J. Cell Sci. 126, 4325–4330.
Mijaljica, D., Prescott, M., Devenish, R.J., 2010. The intricacy of nuclear membrane dynamics during nucleophagy. Nucleus 1, 213–223.
Mijaljica, D., Prescott, M., Devenish, R.J., 2012. A late form of nucleophagy in *Saccharomyces cerevisiae*. PLoS One 7 (6), e40013.
Minibayeva, F., Dmitrieva, S., Ponomareva, A., et al., 2012. Oxidative stress-induced autophagy in plants: the role of mitochondria. Plant Physiol. Biochem. 59, 11–19.
Miracco, C., Meng, G.C., Franchi, A., et al., 2010. Beclin 1 and LC3 autophagic gene expression in cutaneous melanocytic lesions. Hum. Pathol. 41, 503–512.
Mizushima, N., 2007. Autophagy: process and function. Genes Dev. 21, 2861–2973.
Mizushima, N., Kuma, A., 2008. Autophagosomes in GFP-LC3 transgenic mice. Methods Mol. Biol. 445, 119–124.
Mizushima, N., Yoshimori, T., 2007. How to interpret LC3 immunoblotting? Autophagy 3, 542–545.
Mohapatra, P., Preet, R., Das, D., et al., 2012. Quinacrine-mediated autophagy and apoptosis in colon cancer cells is through a p53- and p21-dependent mechanism. Oncol. Res. 20, 81–91.
Momoi, T., 2006. Conformational diseases and ER stress-mediated cell death: apoptotic cell death and autophagic cell death. Curr. Mol. Med. 6, 111–118.
Motley, A.M., Nuttall, J.M., Hettema, E.H., 2012. Pex3-anchored Atg36 tags peroxisomes for degradation in *Saccharomyces cerevisiae*. EMBO J. 31, 2852–2868.
Munro, J.B., Mothes, W., 2015. Structure and dynamics of the native HIV-1 Env trimer. J. Virol. 89, 5752–5755. http://dx.doi.org/10.1128/JV1.03187-14.
Nakai, A., Yamaguchi, O., Takeda, T., et al., 2007. The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress. Nat. Med. 13, 619–624.
Nakajima, H., Hamasaki, T., Tarui, S., 1995. Phosphofructokinase: recent advances in molecular biology. Muscle Nerve 3, S28–S34.
Nemchenko, A., Chiong, M., Turer, A., et al., 2011. Autophagy as a therapeutic target in cardiovascular disease. J. Mol. Cell. Cardiol. 300, H2123–H2134.
Nickel, W., Rabouille, C., 2009. Mechanisms of regulated unconventional protein secretion. Nat. Rev. Mol. Cell Biol. 10, 148–155.
Nicoletti, F., Fagone, P., Meroni, P., et al., 2011. mTOR as a multifunctional therapeutic target in HIV infection. Drug Discov. Today 16, 715–721.
Nixon, R.A., Yang, D.S., 2011. Autophagy failure in Alzheimer's disease – locating the primary defect. Neurobiol. Dis. 43, 38–45.
Novak, I., 2012. Mitophagy: a complex mechanism of mitochondrial removal. Antioxid. Redox Signal. 17, 794–802.
Odagiri, S., Tanji, K., Mori, F., et al., 2012. Autophagic adaptor protein NBR1 is localized in Lewy bodies and glial cytoplasmic inclusions and is involved in aggregate formation in α-synucleinopathy. Acta Neuropathol. 124, 173–186.
Ogata, M., Hino, S., Saito, A., et al., 2006. Autophagy is activated for cell survival after endoplasmic reticulum stress. Mol. Cell Biol. 26, 9220–9231.
Ohsumi, Y., Mizushima, N., 2004. Two ubiquitin-like conjugation systems essential for autophagy. Semin. Cell Develop. Biol. 15, 231–236.
Oka, T., Hikoso, S., Yamaguchi, O., et al., 2012. Mitochondrial DNA that escapes from autophagy causes inflammation and heart failure. Nature 485, 251–256.
Okamoto, K., 2014. Organellophagy: eliminating cellular building blocks via selective autophagy. J. Cell Biol. 205, 435–445.
Olson, M., Kornbluth, S., 2001. Mitochondria in apoptosis and human disease. Curr. Mol. Med. 1, 91–122.
Orhon, I., Dupont, N., Pampliega, O., et al., 2015. Autophagy and regulation of cilia function and assembly. Cell Death Differ. 22, 389–397.
Orvedahl, A., Alexander, D., Talloczy, Z., et al., 2007. HSV-1 1CP34.5 confers neurovirulence by targeting the Beclin 1 autophagy protein. Cell Host Microbe 1, 23–35.
Otera, H., Wang, C., Cleland, M.M., et al., 2010. Mff is an essential factor for mitochondrial recruitment of Drpl during mitochondrial fission in mammalian cells. J. Cell Biol. 191, 1141–1158.

Ouimet, M., Marcel, Y.L., 2012. Regulation of lipid droplet cholesterol efflux from macrophage foam cells. Arterioscler. Thromb. Vasc. Biol. 32, 575–581.
Ouyang, L., Shi, Z., Zhao, S., et al., 2012. Programmed cell death pathways in cancer: a review of apoptosis, autophagy, and programmed necrosis. Cell Prolif. 45, 487–498.
Ovadje, P., Chochkeh, M., Akbari-Asl, P., et al., 2012. Selective induction of apoptosis and autophagy through treatment with dandelion root extract in human pancreatic cancer cells. Pancreas 41, 1039–1047.
Øverbye, A., Fengsrud, M., Seglen, P.O., 2007. Proteomic analysis of membrane-associated proteins from rat liver autophagosome. Autophagy 3, 300–322.
Pagliarini, V., Wirawan, E., Romagnoli, A., et al., 2012. Proteolysis of Ambra1 during apoptosis has a role in the inhibition of the autophagic pro-survival response. Cell Death Differ. 19, 1495–1504.
Palmer, C.S., Osellame, L.D., Latine, D., et al., 2011. MiD49 and Mid51, new components of the mitochondrial fission machinery. EMBO Rep. 12, 565–573.
Papinski, D., Schuschnig, M., Reiter, W., et al., 2014. Early steps in autophagy depend on direct phosphorylation of Atg9 by the Atg1 kinase. Mol. Cell 53, 471–483.
Park, S.-H., Kim, J.-H., Chi, G.Y., et al., 2012. Induction of apoptosis and autophagy by sodium selenite in A549 human lung carcinoma cells through generation of reactive oxygen species. Toxicol. Lett. 212, 252–261.
Perrin, A.J., Jiang, X., Birmingham, C.L., et al., 2004. Recognition of bacteria in the cytosol of mammalian cells by the ubiquitin system. Curr. Biol. 14, 806–811.
Petiot, A., Ogier-Denis, E., Blommaart, E.F., et al., 2000. Distinct classes of phosphatidylinositol 3′-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells. J. Biol. Chem. 275, 992–998.
Pickford, F., Masliah, E., Britschgi, M., 2008. The autophagy-related protein beclin 1 shows reduced expression in early Alzheimer disease and regulates amyloid β accumulation in mice. J. Clin. Invest. 118, 2190–2199.
Prudovsky, I., Mandinova, A., Soldi, R., et al., 2003. The non-classical export routes LFGF1 and IL-1 point the way. J. Cell Sci. 116, 4871–4881.
Railborg, C., Stenmark, H., 2009. The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. Nature 458, 445–452.
Rambold, A.S., Kostelecky, B., Elia, N., et al., 2011a. Tubular network formation protects mitochondria from autophagosomal degradation during nutrient starvation. Proc. Natl. Acad. Sci. U. S. A. 108, 10190–10195.
Rambold, A.S., Kostelecky, B., Lippincott-Schwartz, J., 2011b. Together we are stronger. fusion protects mitochondria from autophagosomal degradation. Autophagy 7 (12), 1568–1569.
Randow, F., 2011. How cells deploy ubiquitin and autophagy to defend their cytosol from bacterial invasion. Autophagy 7 (3), 304–309.
Reichert, A.S., Neupert, W., 2004. Mitochondriomics or what makes us breathe. Trends Genet. 20, 555–562.
Rello-Varona, S., Lissa, D., Shen, S., et al., 2012. Autophagic removal of micronuclei. Cell Cycle 11, 170–176.
Renaud, E., 2010. Protein misfolding and degenerative diseases. Nat. Educ. 3, 28–35.
Roberts, P., Moschitch-Moshkovitz, S., Kvam, E., et al., 2003. Piecemeal microautophagy of nucleus in *Saccharomyces cerevisiae*. Mol. Biol. Cell 14, 129–141.
Romanov, J., Walczak, M., Lbiricu, I., et al., 2012. Mechanism and functions of membrane binding by the Atg5–Atg12/Atg16 complex during autophagosome formation. EMBO J. 31, 4304–4317.
Romisch, K., 2005. Endoplasmic reticulum-associated degradation. Annu. Rev. Cell Develop. Biol. 21, 435–456.
Ron, D., Walter, P., 2007. Signal integration in the endoplasmic reticulum unfolded protein response. Nat. Rev. Mol. Cell Biol. 8, 519–529.
Rong, Y., McPhee, C.K., Deng, S., et al., 2011. Spinster is required for autophagic lysosome reformation and mTOR reactivation following starvation. Proc. Natl. Acad. Sci. U. S. A. 108, 7826–7831.
Rubinszstein, D.C., DiFiglia, M., Heintz, N., et al., 2005. Autophagy and its possible roles in nervous system diseases, damage, and repair. Autophagy 1, 11–22.
Rubio, N., Coupienne, I., Di Valentin, E., et al., 2012. Spatiotemporal autophagic degradation of oxidatively damaged organelles after photodynamic stress is amplified by mitochondrial reactive oxygen species. Autophagy 8, 1312–1324.
Sandberg, M., Borg, L.A.H., 2006. Intracellular degradation of insulin and crinophagy are maintained by nitric oxide and cyclo-oxygenase 2 activity in isolated pancreatic islets. Biol. Cell 98 (5), 307–315.
Sanjuan, M.A., Milasta, S., Green, D.R., 2009. Toll-like receptor signaling in the lysosomal pathways. Immunol. Rev. 227, 203–220.
Sarbassov, D.D., Ali, S.M., Sabatini, D.M., 2005. Growing roles for the mTOR pathway. Curr. Opin. Biol. 17, 596–603.

Sasaki, M., Miyakoshi, M., Sato, Y., et al., 2010. Autophagy mediates the process of cellular senescence characterizing bile duct damages in primary biliary cirrhosis. Lab. Invest. 90, 835–843.
Sato, M., Sato, K., 2012. Maternal inheritance of mitochondrial DNA. Autophagy 8, 424–425.
Sato, M., Sato, K., 2013. Maternal inheritance of mitochondrial DNA by diverse mechanisms to eliminate paternal mitochondrial DNA. Biochim. Biophys. Acta 1833, 1979–1984.
Schrader, M., Bonekamp, N.A., Islinger, M., 2012. Fission and proliferation of peroxisomes. Biochim. Biophs. Acta 1822, 1343–1357.
Schroder, L.A., Ortiz, M.V., Dunn Jr., W.A., 2008. The membrane dynamics of pexophagy are influenced by Sarlp in *Pichia pastoris*. Mol. Biol. Cell 19, 4888–4899.
Schröder, M., Kaufman, R.J., 2005. ER stress and the unfolded protein response. Mutat. Res. 569, 29–63.
Sciarretta, S., Hariharan, N., Monden, Y., et al., 2011. Is autophagy in response to ischemia and reperfusion protective or detrimental for the heart? Pediatr. Cardiol. 32, 275–281.
Segev, N., 2001. Ypt/Rab GTPases: regulators of protein trafficking. Sci. STKE 2001, re11.
Shay, J.W., Roninson, I.B., 2004. Hallmarks of senescence in carcinogenesis and cancer therapy. Oncogene 23, 2919–2933.
Shelly, S., Lukinova, N., Bambina, S., et al., 2009. Autophagy is an essential component of *Drosophila* immunity against vesicular stomatitis virus. Immunity 30, 588–598.
Shen, Y., Li, D.D., Wang, L.L., et al., 2008. Decreased expression of autophagy-related proteins in malignant epithelial ovarian cancer. Autophagy 16, 1067–1068.
Shi, C.S., Kehri, J.H., 2008. MyD88 and Trif target Beclin 1 to trigger autophagy in macrophages. J. Biol. Chem. 283, 33175–33182.
Shintani, T., Huang, W.P., Stromhaug, P.E., et al., 2002. Mechanism of cargo selection in the cytoplasm to vacuole targeting pathway. Dev. Cell 3, 825–837.
Shore, G.C., Papa, F.R., Oakes, S.A., 2011. Signaling cell death from the endoplasmic reticulum stress response. Curr. Opin. Cell Biol. 23, 143–149.
Singh, R., Cuervo, A.M., 2011. Autophagy in the cellular energetic balance. Cell Metab. 13, 495–504.
Singh, R., Cuervo, A.M., 2012. Lipophagy: connecting autophagy and lipid metabolism. Int. J. Cell Biol. 2012, 282041.
Singh, R., Kaushik, S., Wang, Y., et al., 2009. Autophagy regulates lipid metabolism. Nature 458, 1131–1135.
Sir, D., Kuo, C.-F., Tian, Y., et al., 2012. Replication of hepatitis C virus RNA on autophagosomal membranes. J. Biol. Chem. 287, 18036–18043.
Sir, D., Ou, J.H., 2010. Autophagy in viral replication and pathogenesis. Mol. Cells 29, 1–7.
St John, J.C., Schatten, G., 2004. Paternal mitochondrial DNA transmission during nonhuman primate nuclear transfer. Genetics 167, 897–905.
Stasyk, O.V., Nazarko, T.Y., Sibirny, A.A., 2008. Methods of plate pexophagy monitoring and positive selections for ATG gene cloning in yeasts. Methods Enzymol. 451, 229–239.
Subramani, S., Malhotra, V., 2013. Non-autophagic roles of autophagic-related proteins. EMBO Rep. 14, 143–151.
Tainer, J.A., Getzoff, E.D., Alexander, H., et al., 1984. The reactivity of anti-peptide antibodies is a function of the atomic mobility of sites in a protein. Nature, 312–127-134.
Takahashi, Y., Coppola, D., Matsushita, N., et al., 2010. Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. Nat. Cell Biol. 9, 1142–1151.
Tamura, N., Oku, M., Sakai, Y., 2010. Atg8 regulates vacuolar membrane dynamics in a lipidation-independent manner in *Pichia pastoris*. J. Cell Sci. 123, 4107–4116.
Tan, M.L., Ooi, J.P., Ismail, N., et al., 2009. Programmed cell death pathways and current antitumor targets. Pharm. Res. 26, 1547–1560.
Tashiro, Y., Urushitani, M., Inoue, H., et al., 2012. Motor neuron-specific disruption of proteasome, but not autophagy, replicates amyotrophic lateral sclerosis. J. Biol. Chem. 287, 42984–42994.
Taylor, J.M., Brody, K.M., Lockhart, P.J., 2012. Parkin co-regulated gene is involved in aggresome formation and autophagy in response to proteasomal impairment. Exp. Cell Res. 318, 2059–2070.
Thorburn, A., 2008. Apoptosis and autophagy: regulatory connections between two supposedly different processes. Apoptosis 13 (1), 1–9.
Tian, Y., Wang, L.-Y., Ou, J.-H.J., 2015. Autophagy and hepatitis B virus. In: Hayat, M.A. (Ed.), Autophagy: Cancer, Other Pathologies, Immunity, Infection, and Aging Academic Press, New York, NY, pp. 169.

Tinoco Jr., I., Bustamante, C., 2002. The effect of force on thermodynamics and kinetics of single molecule reactions. Biophys. Chem. 101–102, 513–533.
Townsend, K.N., Hughson, L.R., Schlie, K., et al., 2012. Autophagy inhibition in cancer therapy: metabolic considerations for antitumor immunity. Immunol. Rev. 249, 176–194.
Ucar, A., Gupta, S.K., Fiedler, J., et al., 2012. The miRNA-212/132 family regulates both cardiac hypertrophy and cardiomyocyte autophagy. Nat. Commun. 3, 1078.
Urra, H., Dufey, E., Lisbona, F., et al., 2013. When ER stress reaches a dead end. Biochim. Biophys. Acta 1833, 3507–3517.
Uttenweiler, A., Schwarz, H., Neumann, H., et al., 2007. The vacuolar transporter chaperone (VTC) complex is required for microautophagy. Mol. Biol. Cell 18, 166–175.
Uversky, V., Oldfield, C., Dunker, A., 2008. Intrinsically disordered proteins in human disease: introducing the D2 concept. Annu. Rev. Biophys. 37, 215–246.
Uversky, V.N., Gillespie, J.R., Fink, A.L., 2000. Why are "natively" unfolded proteins unstructured under physiological conditions? Proteins 41, 415–427.
Vaccaro, M.I., 2012. Zymophagy: selective autophagy of secretory granules. Int. J. Cell Biol. 2012, 396705.
Valdor, R., Macian, F., 2012. Autophagy and the regulation of the immune response. Pharmacol. Res. 66, 475–483.
van Zutphen, T., van der Klei, I.J., Kiel, J.A., 2008. Pexophagy in *Hansenula polymorpha*. Methods Enzymol. 451, 197–215.
Vander Heiden, M.G., Cantley, L.C., Thompson, C.B., 2009. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 324, 1029–1033.
Veenhuis, M., Douma, A., Harder, W., et al., 1983. Degradation and turnover of peroxisomes in the yeast *Hansenula polymorpha*. Arch. Microbiol. 134, 193–203.
Velikkakath, A.K.G., Nishimora, T., Oita, E., et al., 2012. Mammalian Atg2 proteins are essential for autophagosome formation and important for regulation of size and distribution of lipid droplets. Mol. Biol. Cell 23, 896–909.
Virgin, H.W., Levine, B., 2009. Autophagy genes in immunity. Nat. Immunol. 10, 461–470.
Visagie, M.H., Joubert, A.M., 2011. 2-Methoxyestradiol-bis-sulfamate induces apoptosis and autophagy in a tumorigenic breast epithelial cell line. Mol. Cell. Biochem. 357, 343–352.
Wang, X.-J., Chu, N.-Y., Wang, Q.-H., 2012. Newly synthesized bis-benzimidazole derivatives exerting anti-tumor activity through induction of apoptosis and autophagy. Bioorg. Med. Chem. Lett. 22, 6297–6300.
Wani, W.Y., Boyer-Guittaut, M., Dodson, M., et al., 2015. Regulation of protein post-translational modification. Lab. Invest. 95, 14–25.
Wickner, S., Maurizi, M.R., Gottesman, S., 1999. Posttranslation quality control: folding, refolding, and degradating proteins. Science 286, 1888–1893.
Winslow, A.R., Rubinsztein, D.C., 2011. The Parkinson disease protein α-synuclein inhibits autophagy. Autophagy 7, 429–431.
Wullschleger, S., Loewith, R., Hall, M.N., 2006. TOR signaling in growth and metabolism. Cell 124, 471–484.
Xia, H.G., Zhang, L., Chen, G., et al., 2010. Control of basal autophagy by calpain1 mediated cleavage of ATG5. Autophagy 6 (1), 61–66.
Xie, Z., Klionsky, D.J., 2007. Autophagosome formation: core machinery and adaptations. Nat. Cell Biol. 9, 1102–1109.
Xu, Y., Jagannath, C., Liu, X.D., et al., 2007. Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. Immunity 27, 135–144.
Yahiro, K., Satoh, M., Nakano, M., et al., 2012. Low-density lipoprotein receptor-related protein-1 (LRP1) mediates autophagy and apoptosis caused by *Helicobacter pylori* VacA. J. Biol. Chem. 287, 31104–31115.
Yan, M., Zhu, P., Liu, H.M., et al., 2007. Ethanol induced mitochondria injury and permeability transition pore opening: role of mitochondria in alcoholic liver disease. World J. Gastroenterol. 13, 2352–2356.
Yang, F., Chen, W.D., Deng, R., et al., 2012. Hirsutanol A induces apoptosis and autophagy via reactive oxygen species accumulation in breast cancer MCF-7 cells. J. Pharmacol. Sci. 119, 214–220.
Yang, S., Wang, X., Contino, G., et al., 2011. Pancreatic cancers require autophagy for tumor growth. Genes Dev. 25, 717–729.
Ye, Y.-C., Wang, H.-J., Yu, L., et al., 2012. RIP1-mediated mitochondrial dysfunction and ROS production contributed to tumor necrosis factor alpha-induced L929 cell necroptosis and autophagy. Int. Immunopharmacol. 14, 674–682.
Yorimitsu, T., Klionsky, D.J., 2005. Atg11 links cargo to the vesicle-forming machinery in the cytoplasm to vacuole targeting pathway. Mol. Biol. Cell 16, 1593–1605.

Yoshioka, A., Miyata, H., Doki, Y., et al., 2008. LC3: and autophagosome marker, is highly expressed in gastrointestinal cancer. Int. J. Oncol. 33, 461–468.
You, M., Savaraj, N., Kuo, M.T., et al., 2013. TRAIL induces autophagic protein cleavage through caspase activation in melanoma cell lines under arginine deprivation. Mol. Cell Biochem. 374, 181–190.
Young, A.R.J., Narita, M., Ferreira, M., et al., 2009. Autophagy mediates the mitotic senescence transition. Genes Dev. 23, 798–803.
Yu, W.H., Cuervo, A.M., Kumar, A., et al., 2005. Macroautophagy – a novel beta-amyloid peptide-generating pathway activated in Alzheimer's disease. J. Cell Biol. 171, 87–98.
Yue, Z., 2007. Regulation of neuronal autophagy in axon. Autophagy 3 (2), 139–141.
Yue, Z., Jin, S., Yang, C., et al., 2003. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. Proc. Natl. Acad. Sci. U. S. A. 100, 15077–15082.
Zatloukal, K., Stumptner, C., Fuchsbichler, A., et al., 2002. p62 is a common component of cytoplasmic inclusions in protein aggregation diseases. Am. J. Pathol. 160, 255–263.
Zhan, Y., Gong, K., Chen, C., et al., 2012. P38 MAP kinase functions as a switch in MS-275-induced reactive oxygen species-dependent autophagy and apoptosis in human colon cancer cells. Free Rad. Biol. Med. 53, 532–543.
Zhang, X., Qian, S.-B., 2011. Chaperone-mediated hierarchical control in targeting misfolded proteins to aggresomes. Mol. Biol. Cell 22, 3277–3288.
Zhang, S., Clabough, E.B.D., Sarkar, S., et al., 2010. Deletion of the Huntingtin polyglutamine stretch enhances neuronal autophagy and longevity in mice. PLos Genet. 6, 1–15.
Zhao, X., Li, N., Guo, W., et al., 2004. Further evidence for paternal inheritance of mitochondrial DNA in the sheep (*Ovis aries*). Heredity 93, 399–403.
Zhao, Z., Trackray, L.B., Miller, B.C., et al., 2007. Coronavirus replication does not require the autophagy gene Atg5. Autophagy 3 (6), 581–585.
Zhou, Z., Jiang, X., Liu, D., et al., 2009. Autophagy is involved in influenza a virus replication. Autophagy 5, 321–328.
Zhu, H., Tannous, P., Johnstone, J.L., et al., 2007. Cardiac autophagy is a maladaptive response to hemodynamic stress. J. Clin. Invest. 117, 1782–1793.
Ziv, N.E., Spira, M.E., 1995. Axotomy induced a transient and localized elevation of the free intracellular calcium concentration to the millimolar range. J. Neurophysiol. 74, 2625–2637.
Zoncu, R., Efevan, A., Sabatini, D.M., 2011. mTOR: from growth signal integration to cancer, diabetes and ageing. Nat. Rev. Mol. Cell Biol. 12, 21–35.
Zungu, M., Schisler, J., Willis, M.S., 2011. Regulation of mitochondrial fusion and fission by ubiquitin and small ubiquitin-like modifier and their potential relevance in the heart. Circ. J. 75, 2513–2521.

CHAPTER

2

Role of the Beclin 1 Network in the Cross-Regulation Between Autophagy and Apoptosis

Yangchun Xie, Rui Kang and Daolin Tang

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Abstract

Beclin 1, the mammalian homologue of yeast Atg6, is the subunit of class III phosphatidylinositol 3-kinase (PI3KC3, also termed Vps34 in yeast) complex, which plays a critical role in controlling the mammalian autophagic process in response to physiological and pathological stress. The activity of Beclin 1 in this process is regulated by several factors such as transcriptional control, posttranslational modifications, and protein–protein interactions. The binding of Beclin 1 to positive cofactors (e.g., ATG14, NRBF2, UVRAG, BIF-1, RUBICON, AMBRA1, HMGB1, PINK1, and PARKIN) promotes the formation of Beclin 1-PI3KC3 core complex in the initiation of autophagosome formation in autophagy. In contrast, the binding of Beclin 1 to negative cofactors (e.g., BCL-2 or BCL-XL) prevents the formation of the Beclin 1-PI3KC3 core complex in autophagy. Importantly, Beclin 1-mediated autophagy generally limits apoptosis by inhibiting effector caspase degradation, whereas caspase-mediated cleavage of Beclin 1 in apoptosis prevents against autophagy. Thus, the Beclin 1 network plays a central role in the cross-regulation between autophagy and apoptosis. This chapter focuses on the structure and function of Beclin 1 as an important regulator of autophagy, as well as its role in apoptosis.

INTRODUCTION

Autophagy is an intracellular catabolic process that degrades cytoplasmic material (e.g., unused molecules, damaged organelles, and invaded pathogens) in a lysosome-dependent manner. Three different types of autophagy have been identified: chaperone-mediated autophagy, microautophagy, and macroautophagy (hereafter referred to as autophagy) (Yang and Klionsky, 2010). Autophagy is initiated by the formation of a phagophore, a membrane-sequestering compartment. The phagophore's membrane origins may involve multiple sources such as the Golgi apparatus, endoplasmic reticulum (ER), endosome, mitochondria, and the plasma membrane. Later, the nascent membranes mature into completed double-membrane vesicles termed autophagosomes. Finally, autophagosomes fuse with lysosomes to form autolysosomes for the degradation and recycling of cellular components. Thus, autophagy is generally a programmed cell survival process, although excessive autophagy may promote cell death. Different autophagy-related proteins (ATGs) as well as other regulators can form several protein complexes in autophagy, including induction, nucleation, elongation, fusion, and degradation, to fine tune this complex membrane dynamic process (Xie et al., 2015).

The mammalian autophagy gene *Beclin 1*, also termed *Atg6* in yeast, was cloned in 1999 (Aita et al., 1999). It is widely expressed in many tissues and is primarily localized to cytoplasmic structures and organelles (e.g., ER, mitochondrion, Golgi apparatus, and lysosome). Beclin 1 participates in the regulation of autophagy and plays an important role in the formation of class III phosphatidylinositol 3-kinase (PI3KC3, also termed Vps34 in yeast) core complex. The activity of Beclin 1 in this process is regulated by several factors such as transcriptional control, posttranslational modifications, and protein–protein interactions (Kang et al., 2011). This Beclin 1-PI3KC3 core complex is required in order to activate vesicle nucleation in autophagy. Beclin 1-mediated autophagy is implicated in many physiological and pathological process including development, immunity, tumorigenesis, and neurodegeneration. Beclin 1 dysfunction is involved in the regulation of other processes in addition to autophagy, including cell death and apoptosis. This chapter focuses on the structure and function of Beclin 1 as an important regulator of autophagy, as well as its role in apoptosis.

BECLIN 1 STRUCTURE IN AUTOPHAGY

The BCL-2 family, an important regulator of cell death, is characterized by specific homology regions termed BCL-2 homology domains (BH1, BH2, BH3, and BH4). Beclin 1 contains three distinct functional domains: a BH3 domain at the N terminus (amino acids 114–123), a coiled-coil domain (CCD) in the middle (amino acids 144–269), and a C-terminal evolutionarily-conserved domain (ECD, amino acids 244–337). The activity of Beclin 1 in autophagy is decreased when Beclin 1 interacts with anti-apoptosis BCL-2 family members (e.g., BCL-2 and BCL-XL) by its BH3 domain (Pattingre et al., 2005). The CCD acts as a central interaction platform to engage extra factors (e.g., the activating molecule in Beclin 1-regulated autophagy (AMBRA1), UV radiation resistance associated gene (UVRAG) and ATG14) to activate the Beclin 1-PI3KC3 core complex. The ECD and its nuclear export signal are essential for Beclin 1-mediated tumor suppression. In addition, both the ECD and CCD contribute to the interaction between Beclin 1 and PI3KC3 in the induction of autophagy. However, the detailed mechanism underlying the cooperation between different Beclin 1 domains is unclear.

Like BCL-2 family members, Beclin 1 also has the capability to form a dimer. Beclin 1's self-interaction and dimer formation is mediated by the CCD, but not BH3 domain (Adi-Harel et al., 2010). Importantly, BCL-XL and UVRAG can lead to the switch between Beclin 1 monomer and dimer, suggesting that the quaternary structure of Beclin 1 is regulated by Beclin 1-associated components (Noble et al., 2008). The murine gammaherpesvirus-68 (MHV-68) M11, a viral BCL-2 homologue, has the ability to disrupt Beclin 1 homo-oligomer. Interestingly, the affinity between viral BCL-2 member and Beclin 1 is different from human cells (Noble et al., 2008). Remarkably, the dimer formation of Beclin 1 is not affected by autophagic stimuli such as nutrition starvation, mTOR inhibitor (e.g., rapamycin), and overexpression of PI3KC3 or UVRAG *in vitro* (Adi-Harel et al., 2010). Collectively, although many questions remain unanswered regarding the exact mechanism by which a monomer–dimer switch in Beclin 1 modulates autophagy, structural studies of Beclin 1 have deepened our understanding of autophagy on the molecular level.

TRANSCRIPTIONAL REGULATION OF BECLIN 1 IN AUTOPHAGY

Accumulating evidence suggests that several mechanisms, including those of transcription factors and miRNAs, modulate Beclin 1 expression in autophagy. GA-binding protein (GABP, also known as NRF2 or nuclear respiratory factor 2), an ETS transcription factor, is now recognized as a key transcriptional regulator of the expression of several genes including Beclin 1, which is involved in the formation of Beclin 1-PI3KC3 core complex (Zhu et al., 2014). Splicing of X-box-binding protein 1 mRNA, a crucial signal transducer in the ER stress response, triggers autophagy in endothelial cells via transcriptional activation of Beclin 1. p65, a critical component of the canonical NF-κB pathway, has been shown to induce Beclin 1 expression and subsequent autophagy by binding to Beclin 1's promoter in T cells (Copetti et al., 2009). Additionally, E2F transcription factors function in cell cycle control and directly trans-activate the Beclin 1 promoter during autophagy in the human osteosarcoma cell line.

Recently, posttranscriptional regulators that control Beclin 1 expression and autophagy in several cells have been characterized. As critical posttranscriptional regulators, miRNAs recognize and bind to complementary sequences in the three prime untranslated regions (3′ UTRs) of target mRNAs, usually causing gene silencing. miR-30a has been identified as the first miRNA to inhibit autophagy by downregulation of Beclin 1 expression in human cervical cancer cells (Zhu et al., 2009). Later, miR-376b, miR-216b, and miR-519a have been demonstrated to bind the 3′ UTR of Beclin 1 and consequently decrease the expression of Beclin 1 as well as reduce autophagy in human breast cancer cells, Tenon's fibroblasts, and squamous cell carcinoma cells, respectively. These results support a new treatment development strategy of enhancing chemotherapy sensitivity and apoptosis by modulating miRNA-mediated Beclin 1 expression.

POSTTRANSLATIONAL MODIFICATION OF BECLIN 1 IN AUTOPHAGY

Posttranslational modifications play a critical role in the regulation of ATG activity, which is involved in almost all aspects of autophagy (Xie et al., 2015). Several kinases that trigger Beclin 1 phosphorylation in the induction of autophagy have been identified. Besides to unc-51 like autophagy activating kinase 1 (ULK1)-mediated Beclin 1 phosphorylation on Ser14 in activation of PI3KC3 complex during starvation (Russell et al., 2013), death-associated protein kinase (DAPK)-mediated phosphorylation of Beclin 1 on Thr 119 at the BH3 domain induces the dissociation of BCL-2 from Beclin 1 and subsequent PI3KC3 complex activation in HEK 293 cells (Zalckvar et al., 2009). ROCK1 (Rho-associated, coiled-coil containing protein kinase 1) has been shown to enhance autophagy by directly inducing Beclin 1 phosphorylation on Thr119 and subsequently disrupting the association of Beclin 1-BCL-2 complex in human cervical cancer cells during metabolic stress (Gurkar et al., 2013). In addition, phosphorylation of Beclin 1 at Ser90 and Ser93 can be mediated by ATG14 (also known as Beclin 1-associated autophagy-related key regulator, Barkor) in an indirect manner in HEK 293 cells during starvation-induced autophagy (Fogel et al., 2013).

In starvation-induced autophagy, c-Jun N-terminal kinase 1 (JNK1) phosphorylates BCL-2 at residues Tyr69, Ser70, and Ser87 of the nonstructured loop, reducing its interaction with Beclin 1 and activating autophagy (Wei et al., 2008). However, this process is time-limited in the early period of nutrition deficiency because long-term starvation will result in the disruption of BCL-2-BAX complex and activation of caspase-3, which initiates apoptosis. Moreover, the chromosome protein high mobility group box 1 (HMGB1) promotes the extracellular signal regulated kinase (ERK)-dependent phosphorylation of BCL-2, which leads to the dissociation of Beclin 1 from BCL-2 and the induction of autophagy (Tang et al., 2010). In contrast, the serine/threonine kinase AKT-mediated phosphorylation of Beclin 1 at Ser295 and Ser234 inhibits autophagy through enhanced Beclin 1 binding to intermediate filament proteins such as 14-3-3 and Vimentin in human cancer cells. The epidermal growth factor receptor (EGFR) tyrosine kinase regulates autophagy through phosphorylating Beclin 1 at multisite residues (e.g., Tyr229, Tyr 233, and Tyr352) in nonsmall-cell lung carcinoma cells (Wei et al., 2013). AMP-activated protein kinase (AMPK) activates PI3KC3 complex and induces autophagy by directly phosphorylating Beclin 1 at Ser91 and Ser94 (Kim

et al., 2013). Moreover, MAPKAPK2 and MAPKAPK3, members of the p38 signaling pathway, promote Beclin 1 phosphorylation at Ser90 to mediate starvation-induced autophagy in human cervical cancer cells. This process can be inhibited by BCL-2 *in vitro* and *in vivo*. Thus, the activity of Beclin 1 in autophagy is regulated by phosphorylation of itself as well as its partners.

Additionally, ubiquitination and deubiquitination of Beclin 1 also plays either a positive or negative role in the regulation of autophagy. Tumor necrosis factor receptor-associated factor 6 (TRAF6), an E3 ubiquitin protein ligase, controls K63-linked ubiquitination of Beclin 1 at Lys117, whereas AMBRA1 phosphorylates it at Lys437 to trigger autophagy, suggesting an interplay between ubiquitination and phosphorylation of Beclin 1 (Shi and Kehrl, 2010; Xia et al., 2013). However, WASH (Wiskott–Aldrich syndrome protein and SCAR homologue) can reduce AMBRA1-mediated ubiquitination of Beclin 1 to suppress autophagy (Xia et al., 2013). Moreover, lys11-linked polyubiquitination of Beclin 1 can be triggered by NEDD4 (neural-precursor-cell-expressed developmentally down-regulated 4) in human cervical cancer cells (Platta et al., 2012). The deubiquitinating enzymes ubiquitin carboxyl-terminal hydrolase 10 (USP10) and USP13 contribute to balancing the stability of PI3KC3 complex by controlling the ubiquitination of Beclin 1 (Liu et al., 2011). Collectively, ubiquitination is an important event in fine-tuning the function of Beclin 1 in activation of PI3KC3 complex during autophagy.

BECLIN 1-PI3KC3 CORE COMPLEX IN AUTOPHAGY

PI3Ks are a family of enzymes including class I, class II, and class III/PI3KC3 that are involved in multiple cell processes such as cell growth, proliferation, differentiation, and migration. Notably, PI3KC3 plays an essential role in the induction of autophagy, and inhibition of PI3KC3 activity by small molecular compounds (e.g., wortamannin or 3-methyladenine) can block autophagosome formation. Interestingly, PI3KC3 forms at least three different complexes in yeast and mammalian cells (Feng et al., 2014). Among them, PI3KC3 complex I contains PI3KC3, ATG14, and Beclin 1, which is positively regulated by AMBRA1 or negatively regulated by BCL-2 after binding to Beclin 1 in the induction of autophagy. ATG14 targets the complex to the probable site of autophagosome formation, thereby sorting the complex to function specifically in autophagy (Sun et al., 2008). The interaction between ATG14 and PI3KC3 requires Beclin 1 activity, whereas ATG14 seems not important for the interaction between Beclin 1 and PI3KC3 (Sun et al., 2008). In addition to autophagy, ATG14 is important for the endocytic trafficking pathway by binding to the fusogenic SNARE effector protein Snapin. PI3KC3 complex II substitutes UVRAG for ATG14 and is positively regulated by BIF1 (endophilin B1/BAX-interacting factor 1, also termed SH3GLB1) in both endocytosis and autophagy. UVRAG directly binds the CCD of Beclin 1, although UVRAG has no effect on Beclin 1 self-interaction (Adi-Harel et al., 2010; Liang et al., 2006). Apart from UVRAG-mediated activation of Beclin 1-PI3KC3 complex (Liang et al., 2006), UVRAG also promotes autophagosome maturation by recruitment of the fusion machinery to the late endosome in mammalian cells (Liang et al., 2008). In addition, UVRAG contributes to endosomal trafficking and apoptosis resistance, which is autophagy-independent. The stability of UVRAG protein seems to decrease when Beclin 1 and PI3KC3 are knocked down, suggesting

that UVRAG may be a degradation substrate of autophagy. In the PI3KC3 complex III, BIF1 is replaced by RUBICON (RUN domain protein as Beclin 1-interacting and cysteine-rich containing protein, the negative regulator of UVRAG). BIF1 as an autophagy activator interacts with Beclin 1 through UVRAG (Takahashi et al., 2007). In contrast, RUBICON, which localizes to the endosome and lysosome, negatively regulates PI3KC3 complex activity after binding PI3KC3 and Beclin 1 by the RH domain and CCD, respectively (Sun et al., 2011). Moreover, RUBICON is a component of non-canonical p22 (phox) or CARD9 complexes that regulate oxidative and cytokine responses in activated macrophages, respectively. Both complexes impact the pathogen-specific host defense. Collectively, the activity of PI3KC3 complex in autophagy depends on Belcin 1-binding partners.

BECLIN 1-BCL-2 COMPLEX IN AUTOPHAGY

It is clear that BCL-2 family members such as BCL-2, BCL-XL, and myeloid cell leukemia 1 (MCL-1) have the ability to suppress autophagy by directly binding Beclin 1 via their BH3 domain (Pattingre and Levine, 2006; Shimizu et al., 2004; Tai et al., 2013). In contrast, mutations of their BH3 domain within Beclin 1 or BCL-2 family proteins significantly inhibit the interaction between Beclin 1 and these BCL-2 family proteins. This change leads to the induction of autophagy. The BH3 mimetic ABT-737 also induces autophagy via disruption of BCL2–Beclin 1 interaction in several cells, including fibroblasts and cancer cells (Pedro et al., 2015). BCL-2, MCL-1, and BCL-XL are well known to act as anti-apoptosis BCL-2 family proteins by directly binding the pro-apoptosis BCL-2 family proteins such as BAX, BAK, and BIM. Thus, the formation of Beclin 1-BCL-2 complex logically inhibits the interaction between BCL-2 family proteins. This competitive binding can explain why inhibition of autophagy triggers apoptosis (Boya et al., 2005). However, in some cases, the anti-apoptotic BCL-2 family members do not directly inhibit autophagy by interfering with the Beclin 1 pathway. Instead, the anti-apoptotic BCL-2 family indirectly regulates autophagy by inhibiting the activity of BAX and BAK in myeloid or fibroblast cell lines (Lindqvist et al., 2014). Indeed, the pro-apoptotic BH3-only BCL-2 family proteins (e.g., BNIP3, BAD, NOXA, PUMA, BIMEL, and BIK) have been demonstrated as activators of autophagy in several cells (Sinha and Levine, 2008). However, the exact mechanism of action of pro-apoptotic BH3-only BCL-2 family proteins in autophagy remains unknown. Homo-oligomers of Beclin 1 may provide a platform for further protein–protein interactions including displacement of BCL-2 or BCL-XL by Beclin 1's positive cofactors. As we discussed previously, the phosphorylation of BCL-2 and Beclin 1 by different kinases such as DAPK and MAPK may impair or enhance the formation of Beclin 1-BCL-2 complex in autophagy.

BECLIN 1-BINDING PROTEINS IN AUTOPHAGY

A large number of Beclin 1-binding proteins that influence the autophagy nucleation and maturation processes have recently been identified. In addition to the PI3CK3 and BCL-2 family members we discussed previously, several important Beclin 1-binding partners and their functions are discussed below.

Nuclear Receptor-Binding Factor-2

NRBF-2, also known as co-modulator of PPAR and RXRα-2 (COPR-2), is thought to act as a transcriptional coactivator by binding the AF-2 region of several nuclear hormone receptors (e.g., PPARα, RARα, RARγ, and RXRα). NRBF-2 is a component of Beclin 1-PI3KC3 complex and has been shown to interact with Beclin 1 and ATG14 via its N terminus and microtubule interaction and trafficking domain, respectively (Lu et al., 2014; Zhong et al., 2014). Suppression of NRBF-2 expression by RNAi significantly increases autophagy under starvation conditions in human epithelial cells, suggesting that NRBF-2 is a negative regulator of autophagy. NRBF-2 deficiency also causes increased intracellular phosphatidylinositol-3 phosphate levels and diminished ATG14–PI3KC3 interactions, leading to suppression of PI3KC3 activity and autophagic flux *in vitro* and *in vivo* (Zhong et al., 2014). These findings suggest that NRBF-2 plays an important role in the assembly of PI3KC3 complex I as well as through its activity for autophagy induction.

Epidermal Growth Factor Receptor

EGFR, also termed HER1, is a transmembrane tyrosine kinase that belongs to the HER/ErbB protein family. It is activated via the binding of its specific ligands such as EGF and transforming growth factor α. Aberrant and continuous activation of this receptor is a common feature of human malignancies. EGFR has been shown to interact with numerous proteins, including Beclin 1. Activated EGFR inhibits autophagy by directly binding to Beclin 1, resulting in its tyrosine phosphorylation (Wei et al., 2013). In addition, EGFR-mediated signaling increases the activity of mTOR, which in turn exerts an inhibitory effect on autophagy. Thus, EGFR-mediated autophagy inhibition may contribute to lung cancer progression and resistance to targeted therapy (Wei et al., 2013). However, another study indicates that autophagy inhibition could enhance the anticancer activity of tyrosine kinase inhibitor, suggesting a dual role of autophagy in cancer therapy.

Human Epidermal Growth Factor Receptor 2

The HER2 proto-oncogene encodes a 185 kDa transmembrane, receptor-like glycoprotein with intrinsic tyrosine kinase activity. Unlike EGFR, HER2 lacks an identified ligand. HER2 gene amplification or HER2 protein overexpression has been implicated in the pathogenesis of breast cancer, and this phenomenon is commonly regarded to predict a poor prognosis. HER2 can bind to the ECD of Beclin 1 in breast cancer cells, which contributes to the acquisition of lapatinib resistance (Han et al., 2013). Lapatinib is an FDA-approved drug for the treatment of metastatic breast cancer, which acts by targeting the HER2 receptor. However, lapatinib can disrupt the interaction between Beclin 1 and HER2, which leads to activation of PI3KC3 complex and induction of autophagy (Han et al., 2013). Thus, upregulated autophagy facilitates the lapatinib resistance of HER2-positive breast cancer cells. In other cases, lapatinib-mediated autophagy could inhibit cell proliferation in liver cancer cells. These findings suggest that HER2 status may affect the function of autophagy in the regulation of the anticancer activity of lapatinib.

Endophilin B1/BAX-Interacting Factor 1

BIF-1 is a member of the endophilin B family and was originally identified as a pro-apoptotic BAX-binding protein to regulate BAX conformational change. Thus, loss of BIF-1 inhibits apoptosis, whereas overexpression of BIF-1 promotes apoptosis. It is now well known that BIF-1 is an important regulator of the membrane dynamics of organelles, including Golgi complex, mitochondria, and autophagosome. The expression of BIF-1 is upregulated following autophagic stimuli; this is mediated by inhibition of GSK-3β (Coppola et al., 2011). Importantly, BIF-1 can bind to Beclin 1 and activate PI3KC3 complex to induce autophagosome formation. This process requires UVRAG activity; knockdown of UVRAG inhibits the interaction between BIF-1 and Beclin 1 (Takahashi et al., 2007). Like Beclin 1- and UVRAG-deficient mice, BIF-1-deficient mice exhibit spontaneous tumor growth (Takahashi et al., 2007). These findings confirm that autophagy acts as a tumor suppressor and that autophagy deficiency promotes tumorigenesis.

Autophagy/Beclin 1 Regulator 1

AMBRA1 is a WD40-containing protein expressed during neurodevelopment that is localized to cytoplasmic vesicles. An important regulator of neural tube development, AMBRA1 deficiency causes neural tube defects in mouse embryos, which are associated with impaired autophagy, increased apoptosis, and protein accumulation (Fimia et al., 2007). This indicates that AMBRA1 plays an important role in protein turnover, cell death, and survival. As a positive regulator of autophagy, AMBRA1 is required for activation of PI3KC3 complex by directly binding to Beclin 1. Thus, suppression of AMBRA1 expression inhibits autophagy in response to starvation and rapamycin (Di Bartolomeo et al., 2010). In contrast, the formation of Beclin 1-BCL-2 complex could inhibit AMBRA1-mediated autophagy, suggesting interplay between Beclin 1-binding partners (Strappazzon et al., 2011). A recent study indicates that AMBRA1/Beclin 1-mediated autophagy is required not only for neural tube development, but also for adult neurogenesis by regulation of stem cell survival. The function of AMBRA1 in autophagy in non-neuro cells remains largely unknown.

High Mobility Group Box 1

HMGB1 is normally a non-histone nuclear protein and is released into the extracellular space during stress. After translocation from the nucleus, cytosolic HMGB1 not only has nuclear and extracellular functions, but is also a Beclin 1-binding protein in the induction of autophagy (Tang et al., 2010). Cysteines 23 and 45 of HMGB1 are required for binding to Beclin 1, which leads to the dissociation of Beclin 1 from its inhibitor BCL-2. Knockout of HMGB1 or mutation of C23/45 in several cells could block autophagy activity following treatment with starvation, rapamycin, and oxidative stress. Moreover, several negative regulators (e.g., p53; Livesey et al., 2012) and positive regulators (e.g., ULK1; Huang et al., 2012) are reported to affect the formation of HMGB1-Beclin 1 complex. HMGB1 plays a significant role not only in the cytosol, but also in the nucleus and extracellular space to activate autophagy (Tang et al., 2011). Moreover, HMGB1-dependent autophagy protects

against infection/sterile inflammation and enhances chemotherapy resistance *in vitro* and *in vivo*, suggesting that HMGB1-mediated autophagy plays a functional role in human disease (Sun and Tang, 2014).

Neuronal Isoform of Protein Interaction, Specifically with TC10

The neuronal isoform of protein interaction, specifically with TC10 (nPIST), was originally reported as a glutamate receptor δ2 (GluRδ2)-binding protein by a PDZ domain (Yue et al., 2002). GluRδ2 is selectively expressed on the postsynaptic membrane at parallel fiber-Purkinje neuron synapses in the cerebellum. Mutant mice deficient in GluRδ2 confirm that GluRδ2 plays an essential role *in vivo* in cerebellar Purkinje cell synapse formation. In addition to binding to GluRδ2, nPIST binds the CCD of Beclin 1 to induce autophagic cell death, which leads to the neurodegeneration seen in lurcher mice *in vivo* (Yue et al., 2002). These findings thus contribute to the understanding of the mechanisms of glutamate-mediated neuronal injury by autophagy, as well as molecular mechanisms of synapse formation.

Vacuole Membrane Protein 1

VMP1 is a transmembrane protein localized to intracellular vacuoles that was originally identified in pancreas acinar cells from acute pancreatitis. VMP1-mediated autophagy contributes to the degradation and clearance of zymogen-containing vacuoles in experimental pancreatitis. VMP1 expression is upregulated following nutrition depletion and rapamycin treatment. Suppression of VMP1 expression by RNAi limits the amount of microtubule-associated protein light chain 3 (LC3)-II, which correlates with the extent of autophagosome formation (Ropolo et al., 2007; Vaccaro et al., 2008). In addition to directly targeting to autophagosome membranes along with LC3, VMP1 is a Beclin 1-binding protein. Further studies demonstrate that the hydrophilic domain at the C-terminus of VMP1 is required for binding to the BH3 domain of Beclin 1. Mutation of this domain in VMP1 therefore inhibits LC3-II formation as well as interaction between VMP1 and Beclin 1. Like other positive cofactors, the formation of VMP1-Beclin 1 complex promotes the dissociation of BCL-2 from Beclin 1 and subsequent PI3KC3 complex activation. Collectively, these studies suggest that VMP1 expression is important for the formation of PI3KC3 complex at the autophagosome site during mammalian autophagy.

Signaling Lymphocyte Activation Molecule

SLAM, also known as CD150, is a novel receptor on immune cells, including T and B lymphocytes, macrophages, and dendritic cells. Autophagy is an essential cellular response in the fight against pathogen infection. In addition to acting as a microbial/measles virus sensor, SLAM mediates pathogen-induced autophagy. SLAM is a Beclin 1-binding protein and facilitates the recruitment and activation of PI3KC3 complex in the immune cell killing of Gram-negative bacteria (Berger et al., 2010). Besides autophagy, SLAM participates in phagocytosis by activation of nicotinamide adenine dinucleotide phosphate oxidase. Collectively, SLAM contributes to pathogen clearance by induction of both autophagy and phagocytosis.

IP(3) Receptor

IP(3) receptor (IP_3R) is the primary membrane glycoprotein responsible for the initiation of intracellular calcium (Ca^{2+}) signaling from ER. It was discovered as a developmentally regulated protein missing in cerebellar mutant mice. Recent studies indicate that IP_3R is an important regulator not only for apoptosis, but also for autophagy. Pharmacological and genetic inhibition of IP_3R triggers autophagy, whereas IP_3R agonists inhibit autophagy, suggesting that IP_3R is a negative regulator of autophagy (Vicencio et al., 2009). Remarkably, IP_3R inhibits PI3KC3 complex activity after the binding of IP_3R to Beclin 1. This process can be regulated by overexpression or knockdown of BCL-2 (Vicencio et al., 2009). Although Ca^{2+} is an important signal to trigger autophagy, IP_3R-mediated Ca^{2+} fluxes are irrelevant to the regulation of autophagy. In addition to negatively controlling Beclin 1-PI3KC3 complex activity in mammalian cells, IP_3R inhibits autophagy by sustaining mTOR activity in chicken DT40 B-lymphocyte cells (Khan and Joseph, 2010). The protective effect of a higher autophagic flux in IP_3R-deficient cells may impair the apoptotic response.

PTEN-Induced Putative Kinase 1/PARKIN

PTEN-induced putative kinase 1 (PINK1) is a mitochondrial serine/threonine kinase that plays a critical role in the regulation of mitochondrial morphology and function. PINK1 phosphorylates Parkin, an E3 ubiquitin ligase, and promotes its translocation to injured mitochondria during oxidative stress. The PINK1–PARKIN pathway is considered to protect cells from stress-induced mitochondrial dysfunction by induction of mitophagy, a selective type of autophagy (Geisler et al., 2010). Knockdown of PINK1 and PARKIN by RNAi increases mitochondrial injury and causes cell death. In addition to mitophagy, PINK1 and PARKIN could regulate nonselective autophagy by binding to Beclin 1 in several cells (Lonskaya et al., 2013; Michiorri et al., 2010). Pathogenic PINK1 mutations have been associated with human Parkinson disease. However, only full-length PINK1, not its cleaved or mutant form, interacts with Beclin 1 to induce autophagy (Michiorri et al., 2010). These findings provide insight into the molecular pathology of PINK1 mutations in Parkinson disease.

SURVIVIN

As a member of the apoptosis inhibitors family, survivin is a prominent anti-apoptotic protein by directly binding and inhibiting caspase 3 activity. Overexpression of survivin is observed in fetal development, cancer cell malignancy, and drug resistance. In contrast, efficient downregulation of survivin expression can enhance chemotherapy sensitivity by induction of apoptosis and autophagic cell death. For example, binding of survivin to Beclin 1 promotes autophagy, which contributes to TRAIL (a death receptor ligand)-induced apoptosis in human glioma cells (Niu et al., 2010). YM155, a novel survivin suppressant, has been shown to induce autophagy-dependent DNA damage and death in several cancer cells. However, other studies suggest that survivin-mediated autophagy contributes to drug resistance. Like the ubiquitin–proteasome pathway, autophagy may regulate survivin degradation, suggesting a possible mechanism regulating the cross-talk between apoptosis and autophagy.

Pathogen-Derived Beclin 1 Interaction Partners

Several pathogen-derived proteins such as vBCL-2 (KSHV and murine γHV68 M11, A179L), ICP34.5 (HSV-1), M2 (influenza A), Nef (HIV), E1B19K (adenovirus), and orf16 (KSHV) have been demonstrated to bind Beclin 1 and inhibit autophagosome maturation. These studies indicate that inhibition of autophagosome formation seems to benefit viral replication. In contrast, other viruses appear to use components of the autophagic machinery for viral replication. Thus, autophagy plays a dual role in antiviral defense or pro-viral survival during viral infection.

BECLIN 1-MEDIATED INTERPLAY BETWEEN APOPTOSIS AND AUTOPHAGY

As a stress response, upregulated autophagy has been implicated in protection against harmful stimuli-mediated cell death such as apoptosis. Thus, knockdown of ATGs in many cells leads to increased apoptosis. Unlike the "BH3-only members" of the BCL-2 family, overexpression of Beclin 1 fails to induce apoptosis. In contrast, a number of studies show that Beclin 1 inhibits apoptosis, including the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. Moreover, depletion of Beclin 1 increases cleavage and activation of caspase-3. Although the precise mechanisms of Beclin 1 in apoptosis are currently unknown, data indicate that Beclin 1-mediated autophagy could directly clear apoptotic cells or degrade activated caspase such as caspase-8 (Hou et al., 2010).

Caspases are cysteine aspartyl proteases that serve as the primary mediators of apoptosis. Two types of apoptotic caspases are present in mammalian cells including initiator (caspase-2, 8, 9, 10) and effector (caspase-3, 6, 7) caspases. Notably, several ATGs such as ATG5, Beclin 1, and ATG4 can be cleaved by different caspases, which results in decreased or increased autophagic activity. For example, Beclin 1 can be cleaved by caspase-3, 7, and 8 to form N- and C-terminal fragments (Djavaheri-Mergny et al., 2010; Luo and Rubinsztein, 2010). Moreover, the C-terminal fragments of Beclin 1 can translocate to the mitochondria to induce apoptosis by proapoptotic BCL-2 family members. In addition, loss of HMGB1 inhibits autophagy and increases apoptosis partly by promotion of calpain-mediated Beclin 1 cleavage. This process is important for the inflammatory response due to the fact that autophagy generally inhibits inflammasome activation. Collectively, these studies suggest that both caspases and calpain facilitate Beclin 1 cleavage, leading to impaired autophagy. Further investigation of Beclin 1 cleavage events will increase the understanding of interplay between autophagy and apoptosis in human health and disease.

CONCLUSIONS

Beclin 1 is a core component of Beclin 1-PI3KC3 complex, a lipid-kinase complex involved in autophagosome nucleation. The activity of Beclin 1 is tightly affected by many factors, especially different binding partners. In the presence of BCL-2 and BCL-XL, the pro-autophagic activity of Beclin 1 is repressed. In contrast, a growing number of membranic,

cytosolic, and nuclear proteins have been identified to active autophagy by binding to Beclin 1. In many cases, Beclin 1-mediated autophagy inhibits apoptosis and promotes survival during stress. As a negative feedback loops, both increased caspase and calpain activity in apoptosis can cleave Beclin 1 to inactivate Beclin 1-mediated autophagy. Thus, a complex Beclin 1 interaction network is essential for cross-regulation between autophagy and apoptosis. Much remains to be studied about the structural basis for signal transduction by Beclin 1 complex in autophagy and apoptosis. The Beclin 1-independent autophagic pathway remains largely unknown. Furthermore, development of highly specific small molecule modulators of Beclin 1 that could be therapeutically beneficial in human disease is urgently needed.

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References

Adi-Harel, S., Erlich, S., Schmukler, E., et al., 2010. Beclin 1 self-association is independent of autophagy induction by amino acid deprivation and rapamycin treatment. J. Cell Biochem. 110, 1262–1271.

Aita, V.M., Liang, X.H., Murty, V.V., et al., 1999. Cloning and genomic organization of beclin 1, a candidate tumor suppressor gene on chromosome 17q21. Genomics 59, 59–65.

Berger, S.B., Romero, X., Ma, C., et al., 2010. SLAM is a microbial sensor that regulates bacterial phagosome functions in macrophages. Nat. Immunol. 11, 920–927.

Boya, P., Gonzalez-Polo, R.A., Casares, N., et al., 2005. Inhibition of macroautophagy triggers apoptosis. Mol. Cell Biol. 25, 1025–1040.

Copetti, T., Bertoli, C., Dalla, E., et al., 2009. p65/RelA modulates BECN1 transcription and autophagy. Mol. Cell Biol. 29, 2594–2608.

Coppola, D., Helm, J., Ghayouri, M., et al., 2011. Down-regulation of Bax-interacting factor 1 in human pancreatic ductal adenocarcinoma. Pancreas 40, 433–437.

Di Bartolomeo, S., Corazzari, M., Nazio, F., et al., 2010. The dynamic interaction of AMBRA1 with the dynein motor complex regulates mammalian autophagy. J. Cell Biol. 191, 155–168.

Djavaheri-Mergny, M., Maiuri, M.C., Kroemer, G., 2010. Cross talk between apoptosis and autophagy by caspase-mediated cleavage of Beclin 1. Oncogene 29, 1717–1719.

Feng, Y., He, D., Yao, Z., et al., 2014. The machinery of macroautophagy. Cell Res. 24, 24–41.

Fimia, G.M., Stoykova, A., Romagnoli, A., et al., 2007. Ambra1 regulates autophagy and development of the nervous system. Nature 447, 1121–1125.

Fogel, A.I., Dlouhy, B.J., Wang, C., et al., 2013. Role of membrane association and Atg14-dependent phosphorylation in beclin-1-mediated autophagy. Mol. Cell Biol. 33, 3675–3688.

Geisler, S., Holmstrom, K.M., Skujat, D., et al., 2010. PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. Nat. Cell Biol. 12, 119–131.

Gurkar, A.U., Chu, K., Raj, L., et al., 2013. Identification of ROCK1 kinase as a critical regulator of Beclin1-mediated autophagy during metabolic stress. Nat. commun. 4, 2189.

Han, J., Hou, W., Lu, C., et al., 2013. Interaction between Her2 and Beclin-1 proteins underlies a new mechanism of reciprocal regulation. J. Biol. Chem. 288, 20315–20325.

Hou, W., Han, J., Lu, C., et al., 2010. Autophagic degradation of active caspase-8: a crosstalk mechanism between autophagy and apoptosis. Autophagy 6, 891–900.

Huang, J., Ni, J., Liu, K., et al., 2012. HMGB1 promotes drug resistance in osteosarcoma. Cancer Res. 72, 230–238.

Kang, R., Zeh, H.J., Lotze, M.T., et al., 2011. The Beclin 1 network regulates autophagy and apoptosis. Cell Death Differ. 18, 571–580.
Khan, M.T., Joseph, S.K., 2010. Role of inositol trisphosphate receptors in autophagy in DT40 cells. J. Biol. Chem. 285, 16912–16920.
Kim, J., Kim, Y.C., Fang, C., et al., 2013. Differential regulation of distinct Vps34 complexes by AMPK in nutrient stress and autophagy. Cell 152, 290–303.
Liang, C., Feng, P., Ku, B., et al., 2006. Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG. Nat. Cell Biol. 8, 688–699.
Liang, C., Lee, J.S., Inn, K.S., et al., 2008. Beclin1-binding UVRAG targets the class C Vps complex to coordinate autophagosome maturation and endocytic trafficking. Nat. Cell Biol. 10, 776–787.
Lindqvist, L.M., Heinlein, M., Huang, D.C., et al., 2014. Prosurvival Bcl-2 family members affect autophagy only indirectly, by inhibiting Bax and Bak. Proc. Natl. Acad. Sci. U. S. A. 111, 8512–8517.
Liu, J., Xia, H., Kim, M., et al., 2011. Beclin1 controls the levels of p53 by regulating the deubiquitination activity of USP10 and USP13. Cell 147, 223–234.
Livesey, K.M., Kang, R., Vernon, P., et al., 2012. p53/HMGB1 complexes regulate autophagy and apoptosis. Cancer Res. 72, 1996–2005.
Lonskaya, I., Hebron, M.L., Desforges, N.M., et al., 2013. Tyrosine kinase inhibition increases functional parkin-Beclin-1 interaction and enhances amyloid clearance and cognitive performance. EMBO Mol. Med. 5, 1247–1262.
Lu, J., He, L., Behrends, C., et al., 2014. NRBF2 regulates autophagy and prevents liver injury by modulating Atg14L-linked phosphatidylinositol-3 kinase III activity. Nat. Commun. 5, 3920.
Luo, S., Rubinsztein, D.C., 2010. Apoptosis blocks Beclin 1-dependent autophagosome synthesis: an effect rescued by Bcl-xL. Cell Death Differ. 17, 268–277.
Michiorri, S., Gelmetti, V., Giarda, E., et al., 2010. The Parkinson-associated protein PINK1 interacts with Beclin1 and promotes autophagy. Cell Death Differ. 17, 962–974.
Niu, T.K., Cheng, Y., Ren, X., et al., 2010. Interaction of Beclin 1 with survivin regulates sensitivity of human glioma cells to TRAIL-induced apoptosis. FEBS Lett. 584, 3519–3524.
Noble, C.G., Dong, J.M., Manser, E., et al., 2008. Bcl-xL and UVRAG cause a monomer-dimer switch in Beclin1. J. Biol. Chem. 283, 26274–26282.
Pattingre, S., Levine, B., 2006. Bcl-2 inhibition of autophagy: a new route to cancer? Cancer Res. 66, 2885–2888.
Pattingre, S., Tassa, A., Qu, X., et al., 2005. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell 122, 927–939.
Pedro, J.M., Wei, Y., Sica, V., et al., 2015. BAX and BAK1 are dispensable for ABT-737-induced dissociation of the BCL2-BECN1 complex and autophagy. Autophagy 11, 452–459.
Platta, H.W., Abrahamsen, H., Thoresen, S.B., et al., 2012. Nedd4-dependent lysine-11-linked polyubiquitination of the tumour suppressor Beclin 1. Biochem. J. 441, 399–406.
Ropolo, A., Grasso, D., Pardo, R., et al., 2007. The pancreatitis-induced vacuole membrane protein 1 triggers autophagy in mammalian cells. J. Biol. Chem. 282, 37124–37133.
Russell, R.C., Tian, Y., Yuan, H., et al., 2013. ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase. Nat. Cell Biol. 15, 741–750.
Shi, C.S., Kehrl, J.H., 2010. TRAF6 and A20 regulate lysine 63-linked ubiquitination of Beclin-1 to control TLR4-induced autophagy. Sci. Signal., 3. ra42.
Shimizu, S., Kanaseki, T., Mizushima, N., et al., 2004. Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes. Nat. Cell Biol. 6, 1221–1228.
Sinha, S., Levine, B., 2008. The autophagy effector Beclin 1: a novel BH3-only protein. Oncogene 27 (Suppl. 1), S137–S148.
Strappazzon, F., Vietri-Rudan, M., Campello, S., et al., 2011. Mitochondrial BCL-2 inhibits AMBRA1-induced autophagy. EMBO J. 30, 1195–1208.
Sun, Q., Fan, W., Chen, K., et al., 2008. Identification of Barkor as a mammalian autophagy-specific factor for Beclin 1 and class III phosphatidylinositol 3-kinase. Proc. Natl. Acad. Sci. U. S. A. 105, 19211–19216.
Sun, Q., Zhang, J., Fan, W., et al., 2011. The RUN domain of rubicon is important for hVps34 binding, lipid kinase inhibition, and autophagy suppression. J. Biol. Chem. 286, 185–191.
Sun, X., Tang, D., 2014. HMGB1-dependent and -independent autophagy. Autophagy 10, 1873–1876.
Tai, W.T., Shiau, C.W., Chen, H.L., et al., 2013. Mcl-1-dependent activation of Beclin 1 mediates autophagic cell death induced by sorafenib and SC-59 in hepatocellular carcinoma cells. Cell Death Dis. 4, e485.

Takahashi, Y., Coppola, D., Matsushita, N., et al., 2007. Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. Nat. Cell Biol. 9, 1142–1151.
Tang, D., Kang, R., Livesey, K.M., et al., 2010. Endogenous HMGB1 regulates autophagy. J. Cell Biol. 190, 881–892.
Tang, D., Kang, R., Livesey, K.M., et al., 2011. High-mobility group box 1 is essential for mitochondrial quality control. Cell Metab. 13, 701–711.
Vaccaro, M.I., Ropolo, A., Grasso, D., et al., 2008. A novel mammalian trans-membrane protein reveals an alternative initiation pathway for autophagy. Autophagy 4, 388–390.
Vicencio, J.M., Ortiz, C., Criollo, A., et al., 2009. The inositol 1,4,5-trisphosphate receptor regulates autophagy through its interaction with Beclin 1. Cell Death Differ. 16, 1006–1017.
Wei, Y., Pattingre, S., Sinha, S., et al., 2008. JNK1-mediated phosphorylation of Bcl-2 regulates starvation-induced autophagy. Mol. Cell 30, 678–688.
Wei, Y., Zou, Z., Becker, N., et al., 2013. EGFR-mediated Beclin 1 phosphorylation in autophagy suppression, tumor progression, and tumor chemoresistance. Cell 154, 1269–1284.
Xia, P., Wang, S., Du, Y., et al., 2013. WASH inhibits autophagy through suppression of Beclin 1 ubiquitination. EMBO J. 32, 2685–2696.
Xie, Y., Kang, R., Sun, X., et al., 2015. Posttranslational modification of autophagy-related proteins in macroautophagy. Autophagy 11, 28–45.
Yang, Z., Klionsky, D.J., 2010. Eaten alive: a history of macroautophagy. Nat. Cell Biol. 12, 814–822.
Yue, Z., Horton, A., Bravin, M., et al., 2002. A novel protein complex linking the delta 2 glutamate receptor and autophagy: implications for neurodegeneration in lurcher mice. Neuron 35, 921–933.
Zalckvar, E., Berissi, H., Mizrachy, L., et al., 2009. DAP-kinase-mediated phosphorylation on the BH3 domain of beclin 1 promotes dissociation of beclin 1 from Bcl-XL and induction of autophagy. EMBO Rep. 10, 285–292.
Zhong, Y., Morris, D.H., Jin, L., et al., 2014. Nrbf2 protein suppresses autophagy by modulating Atg14L protein-containing Beclin 1-Vps34 complex architecture and reducing intracellular phosphatidylinositol-3 phosphate levels. J. Biol. Chem. 289, 26021–26037.
Zhu, H., Wu, H., Liu, X., et al., 2009. Regulation of autophagy by a beclin 1-targeted microRNA, miR-30a, in cancer cells. Autophagy 5, 816–823.
Zhu, W., Swaminathan, G., Plowey, E.D., 2014. GA binding protein augments autophagy via transcriptional activation of BECN1-PIK3C3 complex genes. Autophagy 10, 1622–1636.

CHAPTER

3

Role of Sirt1 as a Regulator of Autophagy

Munehiro Kitada, Yoshio Ogura and Daisuke Koya

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Abstract

Sirt1, a NAD^+-dependent deacetylase, has been implicated in cellular processes, including cell survival, metabolism, and adaptation to cellular stress. Autophagy plays an important role in the maintenance of intracellular homeostasis by removing protein aggregates and damaged organelles. Nutrient-sensing pathways include the mammalian target of rapamycin (mTOR), AMP-activated kinase (AMPK), and Sirt1 pathways and regulate autophagy machinery depending on nutrient status. Sirt1 may induce autophagy directly by deacetylating autophagy-related genes (Atg) 5 and 7 and LC3. In addition, Sirt1 deacetylates FOXOs and modulates the expression of autophagy regulatory molecules. Sirt1-deacetylated FOXO1 stimulates the expression of Rab7, a small GTPase that is a crucial factor in the maturation of autophagosomes and endosomes. Sirt1 also deacetylates FOXO3, leading to activation of its transcriptional activity and subsequent Bnip3-mediated autophagy. Furthermore, nutrient-sensing pathways interact with each other, and Sirt1 may indirectly induce autophagy via the activation of AMPK and inhibition of the mTOR pathway. Thus, Sirt1 regulates autophagy machinery through multiple mechanisms.

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INTRODUCTION

Autophagy is a lysosomal degradation pathway that removes protein aggregates and damaged or excess organelles such as mitochondria, leading to the maintenance of intracellular homeostasis and promotion of cellular health under various stress conditions, including hypoxia, endoplasmic reticulum (ER) stress, and oxidative stress (Kroemer et al., 2010). Autophagy plays a crucial role in several organs, especially metabolic organs, and its alteration is involved in the pathogenesis of metabolic and age-related diseases.

Nutrient-sensing pathways that depend on extracellular nutrient conditions are well-conserved among eukaryotes, from yeasts to mammals. Nutrient-sensing pathways include AMP-activated kinase (AMPK), NAD^+-dependent histone deacetylase Sirtuin1 (Sirt1), and mammalian target of rapamycin (mTOR), which are recognized as autophagy regulatory factors under various nutrient conditions (Kroemer et al., 2010). Starvation or energy depletion is the most potent inducer of autophagy. In nutrient-depleted conditions, AMPK and Sirt1 are activated by an increase in intracellular AMP and NAD^+ levels, respectively, leading to the induction of autophagy. However, mTOR is activated by an increase in glucose, amino acid, and insulin levels in excessive nutrient conditions, resulting in the inhibition of autophagy. These nutrient-sensing pathways use posttranslational phosphorylation and acetylation protein modifications to regulate cellular functions, including autophagy, in metabolic organs under both excess and restricted nutrient conditions. In addition, nutrient-sensing pathways, including AMPK, Sirt1, and mTOR, interact and can regulate each other's function to maintain cellular function as a part of environmental adaptation. Therefore, dysregulation of these pathways is associated with the impairment of autophagy, leading to the development of aging and age-related diseases. In this chapter, we specifically focus on the role of Sirt1 in the regulation of autophagy in nutrient-sensing pathways.

CHARACTERISTICS OF SIRT1

Calorie restriction (CR) has a variety of beneficial effects on life span extension and delays the onset of age-related diseases, such as cardiovascular diseases, neurodegenerative disorders, and diabetes. CR is also accepted as the only established experimental antiaging paradigm (Colman et al., 2009; Cohen et al., 2004). As one of the molecules through which CR elicits life span extension or delays age-related diseases, Sir2 (silent information regulator 2), an NAD^+-dependent deacetylase, was initially identified from studies of aging in yeast (Imai et al., 2000). Homologs of Sir2 in higher eukaryotic organisms are known as sirtuins. Sirt1, the most closely related sirtuin to Sir2, is one of the seven sirtuins in mammals. Sirt1 functions as a class III histone deacetylase, binding to NAD^+ and acetyllysine within its protein targets and generating lysine, 2′-*O*-acetyl-ADP-ribose, and nicotinamide as enzymatic products. Nicotinamide acts as a negative-feedback inhibitor of Sirt1. Sirt1 exists in the nucleus and cytoplasm, although Sirt1 is found predominantly in the nucleus, and a large fraction of Sirt1 in the nucleus is associated with euchromatin (Guarente, 2011).

Mammalian Sirt1 promotes chromatin silencing and transcriptional repression through histone deacetylation. Upon recruitment to chromatin, Sirt1 can directly deacetylate

histone (H)4 lysine 16 (H4K16), H3 lysine 9 (H3K9), H3 lysine 14 (H3K14), and H1 lysine 26 (H1K26) (Vaquero et al., 2004; Imai et al., 2000). These deacetylations promote the hypoacetylation of nucleosomal histones and reduce transcriptional activity. Furthermore, histone acetylation and methylation are often coordinately regulated. Histone deacetylation by Sirt1 may also promote alterations in histone methylation. Sirt1 may enhance histone H4 lysine 20 monomethylation (H4K20me) and H3 lysine 9 trimethylation (H3K9me3) and may reduce H3 lysine 79 dimethylation (H3K79me2) (Vaquero et al., 2007).

In addition, more than a dozen nonhistone proteins, including transcription factors and transcriptional coregulatory proteins, serve as substrates for Sirt1. Thereby, the activation of Sirt1 may lead to reduced apoptosis, enhanced mitochondrial biogenesis, the inhibition of inflammation, the regulation of glucose and lipid metabolism and of circadian rhythm, the induction of autophagy, and the adaptation to cellular stress (Guarente, 2011; Kitada et al., 2013). For example, the involvement of NAD^+ in the deacetylation reaction is thought to link Sirt1 deacetylase activity to metabolism. Sirt1 regulates energy metabolism and mediates the longevity effect of CR by promoting gluconeogenesis and repressing glycolysis in the liver via deacetylation of peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1α (PGC-1α), an important promoter of mitochondrial biogenesis. Sirt1 increases insulin sensitivity by modulating insulin signaling. Sirt1 reduces the expression of tyrosine phosphatase 1B (PTP1B), which is the tyrosine phosphatase for the insulin receptor. In addition, Sirt1 may regulate insulin-induced insulin receptor substrate-2 (IRS-2) tyrosine phosphorylation by regulating its acetylation level. Sirt1 is also involved in insulin secretion. The overexpression of Sirt1 in pancreatic islet β-cells increases ATP production by repressing mitochondrial uncoupling protein 2 (UCP2) expression, thereby leading to the closing of ATP-sensitive K^+ channels and to insulin secretion. In addition, Sirt1 inhibits fat storage and increases fatty acid release in white adipose tissue via repression of PPAR-γ and also regulates components of the circadian clock, such as brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like 1 (BMAL1) and Period 2 (PER2). These diverse functions underscore the interconnectedness of protein acetylation, metabolism, circadian rhythms, and aging. Sirt1 is associated with lipid metabolism through the activation of nuclear receptors, including PPAR-α, liver X receptor (LXR), farnesoid X receptor (FXR), and negative regulation of sterol regulatory element-binding protein (SREBP). Furthermore, Sirt1 deacetylates transcription factors such as forkhead box O (FOXO), p53, poly-ADP-ribose polymerase (PARP)-1, hypoxia inducible factor (HIF)-1α and HIF-2α, nuclear factor (NF)-κB, high-mobility group box 1 (HMGB1) (Rabadi et al., 2015), autophagy-related gene (Atg)5, Atg7, and light chain 3 (LC3) to mediate stress resistance, apoptosis, hypoxia, inflammatory signaling, and autophagy as physiological responses to environmental toxicities (Figure 3.1).

AUTOPHAGY

The term "autophagy" is derived from Greek and means "self-eating." Autophagy is highly conserved from yeast to mammals. It is a bulk degradation process involved in the clearance of damaged proteins and organelles. Autophagy works to maintain cell homeostasis under various stress conditions. Three types of autophagy have been identified in cells:

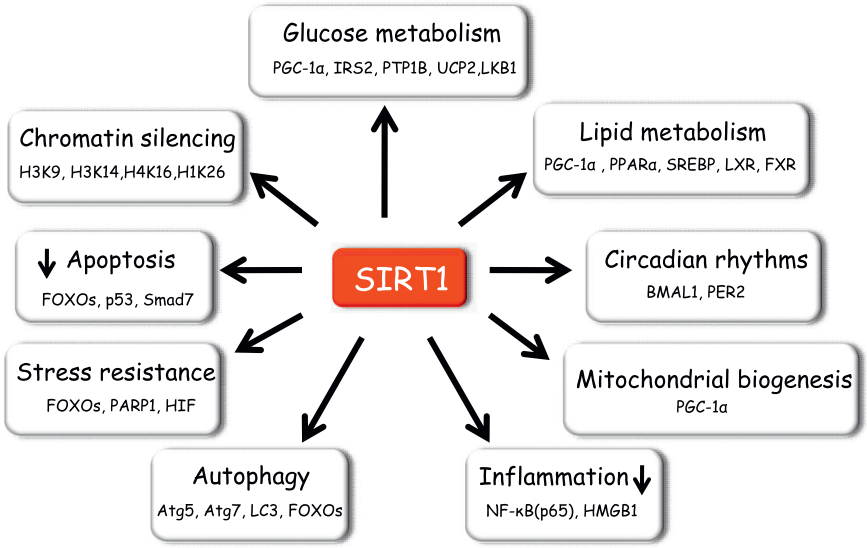

FIGURE 3.1 Biological functions of Sirt1. Sirt1 promotes chromatin silencing through chromatin modifications, mainly deacetylation, and regulates glucose and lipid metabolism, circadian rhythms, mitochondrial biogenesis, stress responses, apoptosis, inflammation and autophagy by deacetylating nonhistone proteins, such as transcription factors and transcriptional coregulatory proteins.

macroautophagy, microautophagy, and chaperone-mediated autophagy. All three types differ in their mechanisms and functions (Kroemer et al., 2010).

During autophagy, *de novo* isolation membranes (phagophores) elongate and fuse while engulfing a portion of the cytoplasm within double-membrane vesicles (autophagosomes). The origin of the autophagosomal membrane is likely the ER membrane (Tooze and Yoshimori, 2010). Five major steps are involved in the formation of autophagosomes: initiation, elongation, closure, fusion, and breakdown. Atgs and proteins are involved in these steps. Autophagy is initiated by the unc-51-like kinase (Ulk) 1 (the mammalian ortholog of yeast Atg1) complex, which comprises Ulk1 serine/threonine protein kinase, Atg13, and FIP200 (the mammalian homolog of yeast Atg17). Phosphorylation of Atg13 and FIP200 by Ulk1 is essential to trigger autophagy. Phagophore nucleation is dependent on Beclin-1 (Atg6 in yeast), an hVps34 or class III phosphatidylinositol 3-kinase (PI3K) complex, which comprises hVps34, hVps15, Beclin-1, and Atg14. During autophagosome elongation/closure, two dependent ubiquitin-like conjugation systems are involved: Atg12 and LC3 (the mammalian ortholog of yeast Atg8). The Atg12–Atg5 conjugate, which forms the Atg12–Atg5–Atg16 complex, contributes to the stimulation and localization of the LC3 conjugation reaction. The cytosolic isoform of LC3 (LC3-I) is conjugated to phosphatidylethanolamine (PE) through two consecutive ubiquitin-like reactions catalyzed by the E1-like enzyme Atg7 and the E2-like enzyme Atg3, forming LC3-II. Thus, LC3-II formation is recognized as a marker of the existence of autophagosomes in cell or animal experiments. After formation, autophagosomes merge with the lysosomal compartment to form autolysosomes.

The protein p62, also known as sequestosome 1, is known to localize to autophagosomes via an interaction with LC3 and to be constantly degraded by the autophagy-lysosome system (Komatsu et al., 2007). The accumulation of p62 is observed in autophagy-deficient cells.

REGULATION OF AUTOPHAGY

Nutrient-Sensing Pathways and Intracellular Stresses Regulate Autophagy Machinery

Autophagy is upregulated in response to nutrient starvation and to extra- or intracellular stress. During nutrient deprivation, autophagosome formation is dramatically induced. Nutrient-sensing pathways, including AMPK, mTOR, and Sirt1, are potent regulators of autophagy. AMPK, which is activated by an increase in intracellular levels of AMP, induces autophagy (Kim et al., 2011).

Conversely, autophagy is inhibited by mTOR, a central cell-growth regulator that integrates growth factor and hypernutrient signals (Kim and Guan, 2015). Understanding the role of Sirt1 in autophagy is still lacking compared with that of AMPK and mTOR; however, recognition of the significance of Sirt1 in the regulation of autophagy has recently been increasing (Ng and Tang, 2013). Recent reports have shown that Sirt1 directly regulates autophagy through deacetylation of autophagy-related molecules and transcriptional factors. In addition, Sirt1 displays cross talk with other nutrition-sensing pathways, such as AMPK and mTOR, thereby Sirt1 indirectly regulates autophagy.

In addition to nutrient starvation, several intracellular stresses, including oxidative stress, hypoxia, and ER stress, can induce autophagy. Reactive oxygen species (ROS) induce autophagy, which removes damaged mitochondria to prevent the accumulation of ROS through multiple mechanisms. The cellular response to an increase in ROS often involves the activation of mitogen-activated protein kinases, including c-jun N-terminal kinase 1 (JNK1), leading to the activation of autophagy (Webber and Tooze, 2010). Wei et al. (2008) demonstrated that JNK1 phosphorylates the Bcl-2 protein, which induces the dissociation of Bcl-2 from Beclin-1 and activates autophagy. ROS can also activate autophagy by stimulating AMPK, which inhibits mTOR. Furthermore, some reports have shown that exogenous hydrogen peroxide can activate PKR-like kinase (PERK), which subsequently phosphorylates eIF2a, oxidizes and activates Atg4 proteases (Liu et al., 2008), and thereby accelerates the production of proteolytic mature LC3 and inhibits mTORC1 activity.

Hypoxia also activates autophagy. Chronic hypoxia causes damage to mitochondria and promotes the intracellular accumulation of ROS. Removing the damaged mitochondria under hypoxic conditions is also an important role of autophagy. In response to hypoxia, HIF-1α transcription factor is activated and induces the transcription of proautophagic BCL2/adenovirus E1V 19-kDa interacting protein 3 (Bnip3) and Bnip3L (NIX). Their protein products compete with Beclin-1 for the binding of BCL2, thereby releasing Beclin-1 and allowing it to induce autophagy (Bellot et al., 2009).

Autophagy also plays an important role in maintenance of the structural and functional integrity of the ER. The ER is involved in protein synthesis and maturation and may also constitute a major source/scaffold of the autophagic isolation membrane (Tooze

and Yoshimori, 2010). The unfolded protein response (UPR), the major ER stress pathway (Buchberger et al., 2010), is a potent stimulus of autophagy. Three sensors located on the membrane of the ER are responsible for monitoring ER stress and initiating the UPR: inositol-requiring ER-to-nucleus signal kinase 1, PERK, and activating transcription factor-6 (ATF6). Among these, PERK and ATF6 act as autophagy inducers (Ogata et al., 2006). PERK mediates the transcriptional activation of the proteins LC3 and Atg5 through the action of the transcription factors ATF4 and CHOP, respectively (Rouschop et al., 2010). PERK may also reduce translation of IκBa, thereby activating NF-κB, which also could contribute to autophagy.

ROLE OF SIRT1 AS A REGULATOR OF AUTOPHAGY

Sirt1 Deacetylates Autophagy-Related Components

Several reports have shown that Sirt1 regulates autophagy through deacetylation of autophagy-related components. Lee et al. (2008) demonstrated an important role for Sirt1 in the regulation of autophagy (Figure 3.2A). According to their report, a transient increase in the expression of Sirt1 is sufficient to stimulate basal rates of autophagy, and Sirt1$^{-/-}$ mouse

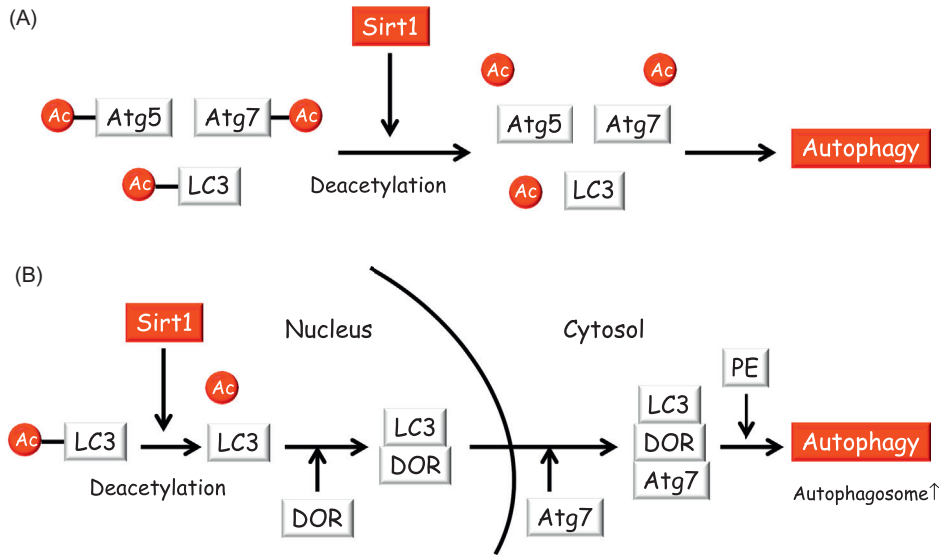

FIGURE 3.2 Sirt1 deacetylates autophagy-related components. (A) Sirt1 can form a molecular complex with several essential components of the autophagy machinery, including Atg5, Atg7, and LC3, and these autophagy components can be directly deacetylated by Sirt1. (B) Deacetylation of a nuclear pool of LC3 by Sirt1 initiates autophagy. Sirt1 deacetylates LC3 upon starvation. Deacetylated LC3 interacts with the nuclear protein DOR, and both proteins relocate from the nucleus to autophagosomes in the cytoplasm. Deacetylated LC3 interacts with Atg7 and other components of the ubiquitin-like conjugation machinery, leading to LC3 conjugation onto phosphatidylethanolamine (PE) and its incorporation into the early autophagosomal membrane.

embryonic fibroblasts do not fully activate autophagy under starvation conditions. In addition, they demonstrated that Sirt1 can form a molecular complex with several essential components of the autophagy machinery, including Atg5, Atg7, and LC3, and these autophagy components can be directly deacetylated by Sirt1. They further showed that Sirt1$^{-/-}$ mice partially resemble Atg5$^{-/-}$ mice, including the accumulation of damaged organelles, disruption of energy homeostasis, accumulation of p62, and early perinatal mortality.

LC3 is a key regulator of autophagy and controls major steps in the autophagy pathway, including autophagosome formation and expansion, cargo recruitment and the fusion of autophagosomes with lysosomes. LC3 is conjugated to the lipid PE by a ubiquitin-like conjugation system involving the E1-like activating enzyme Atg7 and the E2-like conjugating enzyme Atg3. Although LC3 is abundant in the nucleus, LC3 primarily functions in the cytoplasm at sites of autophagosome formation. Nuclear LC3 may serve as a reserve for the cytoplasmic pool and shift to the cytosol when soluble cytoplasmic LC3 is transformed to the lipidated type and is incorporated into autophagic membranes. The regulation of nuclear LC3 redistribution to the cytoplasm and its coordination with LC3 conjugation to autophagic membranes is unclear. Recently, Huang et al. (2015) demonstrated an acetylation/deacetylation-dependent mechanism for the nucleocytoplasmic transport and activation of LC3 in starvation-induced autophagy. Deacetylation of LC3 at K49 and K51 by Sirt1 allows LC3 to interact with the nuclear protein DOR and to return to the cytoplasm with DOR, where it is able to bind Atg7 and other autophagy factors and undergo PE conjugation to preautophagic membranes (Figure 3.2B). The association of deacetylated LC3 with autophagic factors shifts the distribution of LC3 from the nucleus toward the cytoplasm. Although the acetylation of LC3 in full-nutrient conditions maintains it in an inactive state, the activation of Sirt1 during starvation rapidly deacetylates and activates nuclear LC3, leading to the induction of autophagy. Overall, these data identify nuclear activation and nucleocytoplasmic shuttling of LC3 as a novel mechanism that directs autophagy induction upon starvation.

Sirt1 Regulates Autophagy Through Deacetylation of Stress-Responsive Transcription Factors, Including FOXO Family Members

FOXO transcription factors have important roles in many cellular functions, including autophagy. Sirt1 can regulate autophagy indirectly through the deacetylation of the FOXO family members FOXO1 and FOXO3 (Figure 3.3A and B). During glucose starvation, Sirt1 induces the deacetylation of FOXO1 and its activation and nuclear translocation in cardiomyocytes (Hariharan et al., 2010). FOXO1 activation stimulates the expression of Rab7, a small GTPase that is a crucial factor in the maturation of autophagosomes and autolysosomes, including their fusion with lysosomes (Ao et al., 2014). Knockdown of Rab7 inhibits FOXO1-induced autophagy, whereas the overexpression of Rab7 stimulates autophagy (Sengupta et al., 2009). Moreover, interaction between Sirt1 and FOXO1 is also required in starvation-induced autophagy in murine heart.

Hypoxia-induced autophagy largely depends on HIF-1α-induced overexpression of Bnip3 (Bellot et al., 2009). However, although Bnip3 expression is essential for inducing autophagy under hypoxic conditions, Bnip3 is positively regulated by FOXO3, rather than by HIF-1α. Kume et al. found that the reduction of chronic hypoxia-induced autophagy by reduced Sirt1 activation may play a role in the pathogenesis of the aging kidney and that

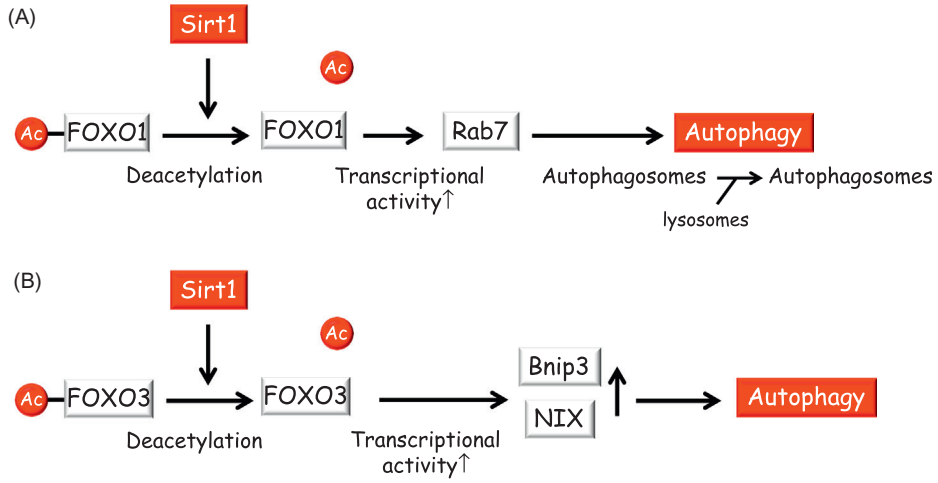

FIGURE 3.3 Sirt1 regulates autophagy by deacetylating stress-responsive transcription factors, including FOXO family members. (A) Sirt1 induces the deacetylation, activation, and nuclear translocation of FOXO1. FOXO1 activation stimulates the expression of Rab7, leading to the maturation of autophagosomes and autolysosomes via fusion with lysosomes. (B) Sirt1 activation deacetylates and activates FOXO3 transcriptional activity and subsequent Bnip3-mediated autophagy.

CR-mediated Sirt1 activation deacetylates and activates FOXO3 transcriptional activity and subsequent Bnip3-mediated autophagy, even in aged kidneys (Kume et al., 2010). In addition, some reports have demonstrated that Sirt1 can deacetylate FOXO3 and FOXO4 (van der Horst et al., 2004) in response to oxidative stress and improve cellular stress resistance, possibly suggesting that this effect was exerted by the induction of autophagy.

Sirt1 Regulates Autophagy by Altering Activation and Interactions Between the AMPK and mTOR Pathways

During nutrient deprivation, autophagosome formation is dramatically induced. However, autophagy is suppressed during excess nutrition states. In both yeast and mammalian cells, two well-characterized signaling cascades that sense nutrient status, the mTOR and AMPK pathways, are potent regulators of autophagy. Sirt1 may regulate autophagy via modulation of mTOR and AMPK activity (Figure 3.4).

mTOR is a serine/threonine protein kinase that is a central regulator of cell growth, proliferation, and metabolism in response to environmental conditions (Laplante and Sabatini, 2012). mTOR forms two functional complexes, termed mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is a rapamycin-sensitive protein kinase complex and consists of mTOR, regulatory-associated protein of mTOR (Raptor), LST8/G-protein β-subunit-like protein (mLST8/GβL), PRAS40, DEP-domain-containing mTOR-interacting protein (Deptor), and Tti1/Tel2. mTORC1 integrates nutrition status and signaling networks, including insulin and insulin-like growth factor-1 (IGF-1) (Jewell and Guan, 2013). TSC1/2 is arguably the most important upstream negative regulator of mTORC1. Insulin

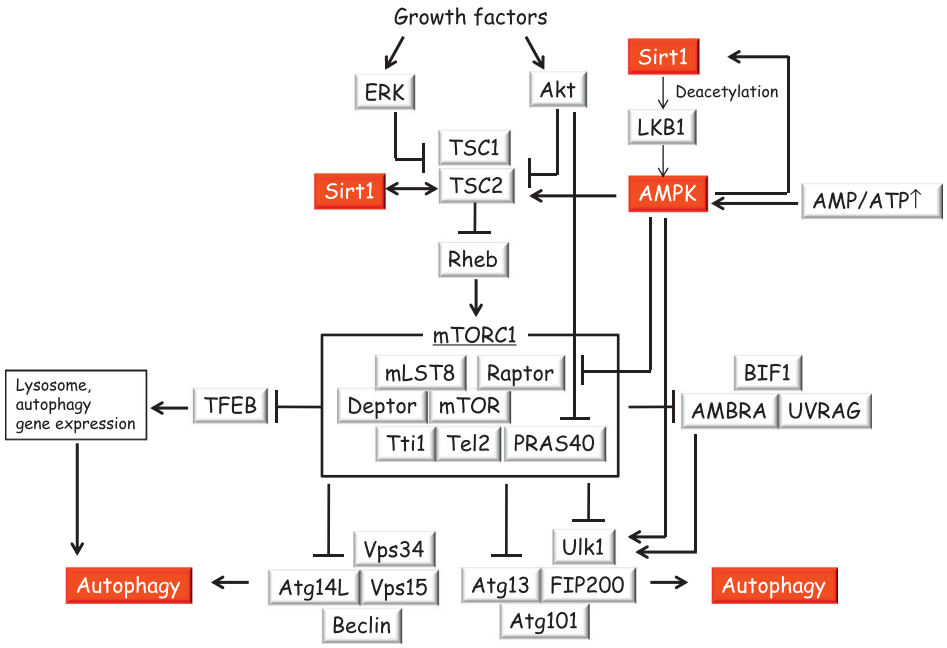

FIGURE 3.4 mTOR signaling and cross talk between AMPK and Sirt1 in the regulation of autophagy. Activation of mTORC1 leads to the inhibition of autophagy through the phosphorylation of multiple autophagy-related proteins, such as Ulk1, Atg13, AMBRA and Atg14L. mTORC1 also phosphorylates and prevents the nuclear localization of the transcription factor TFEB, a regulator of lysosomal and autophagy gene expression. Ulk1 is also directly activated by AMPK, leading to autophagy. Growth factors activate Akt and ERK signaling cascades, resulting in inhibition of the TSC complex. The TSC complex functions as an effector of the Rheb GTPase, leading to activation of mTORC1. AMPK suppresses mTORC1 through activation of the TSC complex or through direct phosphorylation of Raptor. Sirt1 activates AMPK by deacetylating liver kinase B1 (LKB1), whereas AMPK also activates Sirt1. Thus, Sirt1 may activate autophagy by the inhibition of mTORC1 via AMPK activation. In addition, Sirt1 interacts with TSC2 and inhibits mTORC1 activation, resulting in autophagy activation.

signals phosphorylate protein kinase B (Akt) via PI3K and phosphoinositide-dependent kinase 1. Phosphorylated Akt suppresses tuberous sclerosis complex 2 (TSC2), a negative regulator of a GTPase-activating protein (GAP) for the Ras homolog enriched in brain (Rheb) GTPase. Because Rheb is an upstream activator of mTORC1, inhibition of TSC2 results in mTORC1 activation. Akt also directly phosphorylates and inhibits PRAS40, which is a negative regulatory component of mTORC1. Furthermore, activation of extracellular signal-regulated kinase (ERK) by growth factor stimulation phosphorylates TSC2 and inhibits its activity, resulting in the activation of mTORC1.

The activation of mTORC1 by nutrients and growth factors inhibits autophagy through the phosphorylation of multiple autophagy-related proteins, such as Ulk1 (Ser758 (Ser757 in mouse)), Atg13, antiactivating molecule in Beclin-1-regulated *autophagy* (AMBRA) and Atg14L, which promote autophagy initiation and autophagosome nucleation. mTOR also phosphorylates and prevents nuclear transcription of the transcription factor TFEB, a master

regulator of lysosomal and autophagy gene expression. The inhibition of mTORC1 activation leads to the induction of autophagy machinery (Kim et al., 2011, 2013; Kim and Guan, 2015; Nazio et al., 2013; Yuan et al., 2013; Settembre et al., 2011, 2013).

AMPK, which is upregulated by an increase in intracellular AMP/ATP ratios during conditions of low energy levels, such as glucose deprivation, induces autophagy. AMPK phosphorylates TSC2 and increases the TSC1/2 GAP activity, resulting in the suppression of mTORC1 (Inoki et al., 2003). In addition, AMPK phosphorylates Raptor and leads to a decrease in mTORC1 activity through allosteric inhibition (Gwinn et al., 2008). Thus, AMPK activates autophagy through the inhibition of the mTORC1 pathway. In addition to the inhibition of mTORC1, AMPK positively controls the initiation of autophagy by direct Ulk1 phosphorylation (Ser317 and Ser777) (Kim et al., 2011).

Sirt1 links mTORC1 and AMPK and can regulate their activity. Price et al. demonstrated a direct link between Sirt1 and the metabolic benefits of resveratrol, a Sirt1 activator (Price et al., 2012). They reported that a moderate dose of resveratrol first activates Sirt1 and then induces deacetylation of LKB1 and the activation of AMPK, leading to beneficial cellular effects, such as increased mitochondrial biogenesis and function. On the other hand, Ghosh et al. (2010) showed that Sirt1 may negatively regulate mTOR signaling through an interaction with the TSC1/2 complex. We have also previously reported that Sirt1 inactivation may impair autophagy through alterations of mTORC1 and through AMPK activation in THP-1 (human monocytes) (Takeda-Watanabe et al., 2012). Inactivation of Sirt1 using the chemical inhibitors sirtinol and EX-527 activates mTORC1 and decreases AMPK activation, resulting in the suppression of autophagy and increasing inflammation. Thus, these results indicate that Sirt1 positively regulates autophagy via interaction with mTORC1 and AMPK in response to nutrient conditions.

CONCLUDING REMARKS

The understanding of Sirt1 has expanded from the original description of a single NAD^+-dependent class III histone deacetylase that can control the life span of yeast. Sirt1 deacetylates not only histones but also many transcriptional regulators, thereby modulating diverse biological processes, including autophagy. CR-mediated Sirt1 activation has beneficial effects on life span extension, and its activation of autophagy may be implicated in the delay of age-related diseases. Sirt1 also alters other nutrient-sensing pathways, including mTORC1 and AMPK. Therefore, pharmacological or physiological Sirt1 activators should be a novel tool for the regulation of autophagy, which is involved in longevity and in the suppression of age-related diseases. In this chapter, we summarized the function of Sirt1 in the regulation of autophagy. However, because the detailed mechanisms by which Sirt1 regulates the autophagy machinery have not yet been determined, further studies are necessary to provide insight into the exact mechanisms.

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References

Ao, X., Zou, L., Wu, Y., 2014. Regulation of autophagy by the Rab GTPase network. Cell Death Differ. 21, 348–358.
Bellot, G., Garcia-Medina, R., Gounon, P., et al., 2009. Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains. Mol. Cell Biol. 29, 2570–2581.
Buchberger, A., Bukau, B., Sommer, T., 2010. Protein quality control in the cytosol and the endoplasmic reticulum: brothers in arms. Mol. Cell. 40, 238–252.
Cohen, H.Y., Miller, C., Bitterman, K.J., et al., 2004. Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. Science 305, 390–392.
Colman, R.J., Anderson, R.M., Johnson, S.C., et al., 2009. Caloric restriction delays disease onset and mortality in rhesus monkeys. Science 325, 201–204.
Ghosh, H.S., McBurney, M., Robbins, P.D., 2010. SIRT1 negatively regulates the mammalian target of rapamycin. PLoS One 5, e9199.
Guarente, L., 2011. Franklin H. Epstein lecture: sirtuins, aging, and medicine. N. Engl. J. Med. 364, 2235–2244.
Gwinn, D.M., Shackelford, D.B., Egan, D.F., et al., 2008. AMPK phosphorylation of raptor mediates a metabolic checkpoint. Mol. Cell. 30, 214–226.
Hariharan, N., Maejima, Y., Nakae, J., et al., 2010. Deacetylation of FoxO by Sirt1 plays an essential role in mediating starvation-induced autophagy in cardiac myocytes. Circ. Res. 107, 1470–1482.
Huang, R., Xu, Y., Wan, W., et al., 2015. Deacetylation of nuclear LC3 drives autophagy initiation under starvation. Mol. Cell. 57, 456–466.
Imai, S., Armstrong, C.M., Kaeberlein, M., et al., 2000. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature 403, 795–800.
Inoki, K., Zhu, T., Guan, K.L., 2003. TSC2 mediates cellular energy response to control cell growth and survival. Cell 115, 577–590.
Jewell, J.L., Guan, K.L., 2013. Nutrient signaling to mTOR and cell growth. Trends Biochem. Sci. 38, 233–242.
Kim, J., Kundu, M., Viollet, B., et al., 2011. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat. Cell Biol. 13, 132–141.
Kim, Y.C., Guan, K.L., 2015. mTOR: a pharmacologic target for autophagy regulation. J. Clin. Invest. 125, 25–32.
Kim, J., Kim, Y.C., Fang, C., et al., 2013. Differential regulation of distinct Vps34 complexes by AMPK in nutrient stress and autophagy. Cell 152, 290–303.
Kitada, M., Kume, S., Kanasaki, K., et al., 2013. Sirtuins as possible drug targets in type 2 diabetes. Curr. Drug Targets 14, 622–636.
Komatsu, M., Waguri, S., Koike, M., et al., 2007. Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. Cell 131, 1149–1163.
Kroemer, G., Mariño, G., Levine, B., 2010. Autophagy and the integrated stress response. Mol. Cell. 40, 280–293.
Kume, S., Uzu, T., Horiike, K., et al., 2010. Calorie restriction enhances cell adaptation to hypoxia through Sirt1-dependent mitochondrial autophagy in mouse aged kidney. J. Clin. Invest. 120, 1043–1055.
Laplante, M., Sabatini, D.M., 2012. mTOR signaling in growth control and disease. Cell 149, 274–293.
Lee, I.H., Cao, L., Mostoslavsky, R., et al., 2008. A role for the NAD-dependent deacetylase Sirt1 in the regulation of autophagy. Proc. Natl. Acad. Sci. U.S.A. 105, 3374–3379.
Liu, L., Wise, D.R., Diehl, J.A., et al., 2008. Hypoxic reactive oxygen species regulate the integrated stress response and cell survival. J. Biol. Chem. 283, 31153–31162.
Ng, F., Tang, B.L., 2013. Sirtuins' modulation of autophagy. J. Cell. Physiol. 228, 2262–2270.
Nazio, F., Strappazzon, F., Antonioli, M., et al., 2013. mTOR inhibits autophagy by controlling ULK1 ubiquitylation, self-association and function through AMBRA1 and TRAF6. Nat. Cell Biol. 15, 406–416.
Ogata, M., Hino, S., Saito, A., et al., 2006. Autophagy is activated for cell survival after endoplasmic reticulum stress. Mol. Cell Biol. 26, 9220–9231.
Price, N.L., Gomes, A.P., Ling, A.J., et al., 2012. SIRT1 is required for AMPK activation and the beneficial effects of resveratrol on mitochondrial function. Cell Metab. 15, 675–690.
Rabadi, M.M., Xavier, S., Vasko, R., et al., 2015. High-mobility group box 1 is a novel deacetylation target of Sirtuin1. Kidney Int. 87, 95–108.
Rouschop, K.M., van den Beucken, T., Dubois, L., et al., 2010. The unfolded protein response protects human tumor cells during hypoxia through regulation of the autophagy genes MAP1LC3B and ATG5. J. Clin. Invest. 120, 127–141.

Sengupta, A., Molkentin, J.D., Yutzey, K.E., 2009. FoxO transcription factors promote autophagy in cardiomyocytes. J. Biol. Chem. 284, 28319–28331.
Settembre, C., Di Malta, C., Polito, V.A., et al., 2011. TFEB links autophagy to lysosomal biogenesis. Science 332, 1429–1433.
Settembre, C., Fraldi, A., Medina, D.L., et al., 2013. Signals from the lysosome: a control centre for cellular clearance and energy metabolism. Nat. Rev. Mol. Cell Biol. 14, 283–296.
Takeda-Watanabe, A., Kitada, M., Kanasaki, K., et al., 2012. SIRT1 inactivation induces inflammation through the dysregulation of autophagy in human THP-1 cells. Biochem. Biophys. Res. Commun. 427, 191–196.
Tooze, S.A., Yoshimori, T., 2010. The origin of the autophagosomal membrane. Nat. Cell Biol. 12, 831–835.
Vaquero, A., Scher, M., Lee, D., et al., 2004. Human SirT1 interacts with histone H1 and promotes formation of facultative heterochromatin. Mol. Cell. 16, 93–105.
Vaquero, A., Scher, M., Erdjument-Bromage, H., et al., 2007. SIRT1 regulates the histone methyl-transferase SUV39H1 during heterochromatin formation. Nature 450, 440–444.
van der Horst, A., Tertoolen, L.G., de Vries-Smits, L.M., et al., 2004. FOXO4 is acetylated upon peroxide stress and deacetylated by the longevity protein hSir2(SIRT1). J. Biol. Chem. 279, 28873–28879.
Webber, J.L., Tooze, S.A., 2010. Coordinated regulation of autophagy by p38alpha MAPK through mAtg9 and p38IP. EMBO J. 29, 27–40.
Wei, Y., Sinha, S., Levine, B., 2008. Dual role of JNK1-mediated phosphorylation of Bcl-2 in autophagy and apoptosis regulation. Autophagy 4, 949–951.
Yuan, H.X., Russell, R.C., Guan, K.L., 2013. Regulation of PIK3C3/VPS34 complexes by MTOR in nutrient stress-induced autophagy. Autophagy 9, 1983–1995.

CHAPTER

4

Apoptosis Blocks Beclin 1-Dependent Autophagosome Synthesis

Shouqing Luo

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Abstract

Macroautophagy (hereafter referred to as autophagy) is a lysosome-dependent cellular process that mediates the clearance of aberrant intracellular components. The autophagy process includes autophagosome synthesis, autophagosome–lysosome fusion, and cargo degradation. During developmental, physiological, and pathological conditions, cells utilize autophagy to remove cytoplasmic toxic components and maintain energy and nutrition supply, or commit suicide by programmed cell death, such as apoptosis. Although autophagy and apoptosis are interdependent cellular processes, and can cause diverse consequences to cell fate, both of them are evolutionarily conserved cellular mechanisms, of which signaling pathways are connected through various interplays. The cross talk between apoptosis and autophagy has been thought to regulate cell survival or cell death in response to stress signals at cell or organism levels. Here we discuss various mechanisms by which apoptosis negatively regulates canonical or Beclin 1-dependent autophagosome biogenesis and autophagy, and define the paradigms of the role of apoptosis in the process.

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INTRODUCTION

The cells in a metazoan are highly coordinating individual members where sophisticated programs are encoded so that a whole organism can function in an appropriate order. For example, the number of cells within an organism is precisely controlled. Superfluous cells are capable of committing suicide by activating an inherent death program, of which the process is termed programmed cell death. Programmed cell death confers an organism to eliminate unwanted cells during developmental and normal physiological conditions (Bender et al., 2012; Vercammen et al., 2007). Apoptosis is the best-studied mode of programmed cell death. The molecular events involved in apoptosis regulation have been well characterized. Apoptosis is a form of cell death mediated by caspase activation, and it can be categorized into intrinsic and extrinsic pathways, based on signaling origins (Barnhart et al., 2003; Tait and Green, 2010). Intrinsic or mitochondrion-dependent apoptosis is regulated by Bcl-2 family proteins. Bcl-2 family proteins can be divided into three groups, namely, Bax/Bak, Bcl-2-like proteins, and BH3-only proteins (Youle and Strasser, 2008). In these three groups of proteins, only Bax/Bak directly induces apoptosis. Activated Bax or Bak forms a channel in the outer membrane of a mitochondrion and results in mitochondrial outer membrane permeabilization, enabling the release of pro-apoptotic factors including cytochrome *c* that stimulates the assembly of apoptosomes, and in turn results in activation of caspases and apoptosis (Riedl and Salvesen, 2007; Tait and Green, 2010). Bcl-2 inhibits apoptosis by suppressing Bax/Bax activity. BH3-only proteins, including a large number of proteins such as Bim, tBid, PUMA, Noxa, kill cells either by binding Bcl-2 proteins to inhibit its activity or by binding Bax or Bak to activate them (Shamas-Din et al., 2011; Youle and Strasser, 2008). Extrinsic pathways are typified by death ligand-induced apoptotic pathways (Barnhart et al., 2003) (Figure 4.1).

Autophagy is a catabolic process that involves the engulfment of cargo, such as long-lived proteins, intracellular pathogens, and damaged organelles, within double-membrane vesicles, namely autophagosomes. The autophagy process includes autophagosome biogenesis, autophagosome–lysosome fusion, and cargo degradation (Luo and Rubinsztein, 2007) (Figure 4.2). mTOR senses cellular nutrient status (Lum et al., 2005). Under nutrient-replete conditions, mTOR inhibits ULK1/Atg1 activity (Hosokawa et al., 2009). Under starvation conditions, suppression of mTOR activity results in the activation of ULK1 and downstream signals, thus initiates autophagosome synthesis (Kim et al., 2011) (Figure 4.2). Autophagosome biogenesis begins with phagophore nucleation step, in which membranes first form pre-autophagosomal structures. Beclin 1 positively regulates the activity of the class III PI-3 kinase, Vps34 that generates phosphatidylinositol-3-phosphate (PtdIns3P) required for phagophore nucleation (Itakura et al., 2008). Autophagosome elongation involves two essential ubiquitin-like (UBL) conjugations: the conjugation of the UBL protein Atg12–Atg5, and the conjugation of the UBL protein LC3 to phosphatidylethanolamine (PE). Atg5–Atg12 conjugation is catalyzed sequentially by E1-like enzyme Atg7 and E2-like enzyme Atg10 (Mizushima et al., 1998). The Atg5–12 conjugate (Atg5–12) is an E3 ligase to catalyze LC3-PE (LC3-II) conjugation (Hanada et al., 2007; Noda et al., 2013; Otomo et al., 2013) and is required for LC3-II localization to pre-autophagosomal membranes to enable expansion of the membranes (Kaufmann et al., 2014; Romanov et al., 2012; Suzuki et al., 2001), which eventually expand to form complete double-membrane autophagsosomes

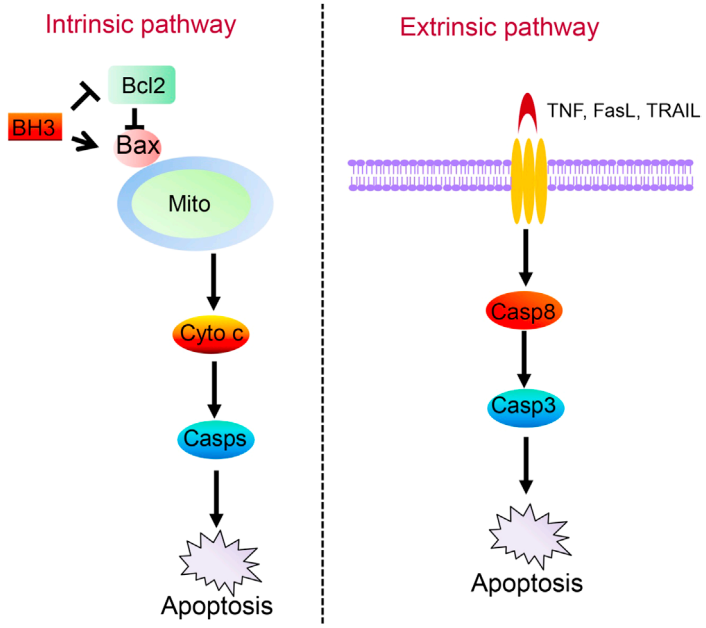

FIGURE 4.1 Apoptosis paradigms. Intrinsic apoptosis pathway is regulated by Bcl-2 family proteins. Activated Bax/Bak induces cytochrome C release and in turn activates caspase-9 and executioner caspases such as caspase-3. Extrinsic apoptosis pathway is initiated by death ligands via ligation to their receptors, resulting in activation of caspase-8 and executioner caspases.

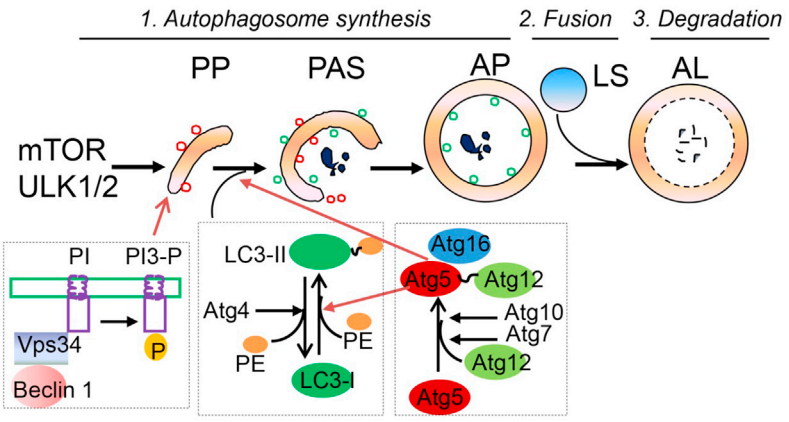

FIGURE 4.2 The autophagy process. The autophagy process includes autophagosome biogenesis, autophagosome–lysosome fusion, and cargo degradation. The Beclin 1–Vps34 complex generates PI3-P that is required for phagophore nucleation. The Atg5–12 complex (with Atg16) is required for LC3-PE (LC3-II) conjugation and LC3-II autophagosomal membrane localization. PP, phagophore; PAS, pre-autophagosomal structure; AP, autophagosome; LS, lysosome; AL, autolysosome; green circle, LC3-II; red circle, Atg5–12/Atg16 complex.

(Figure 4.2). The autophagosomes are subsequently fused with the lysosomes where their contents are degraded and released into cytosol as a supply of nutrition (Levine and Kroemer, 2008). Autophagy is believed to have pro-survival function mainly because of its degradation of aberrant cytoplasmic components to replenish nutrition and energy for cells. Owing to its wide implications under physiological and pathological conditions, such as neurodegenerative diseases and cancer, autophagy has attracted increasing attention in recent years.

Beclin 1 is essential for autophagosome formation in canonical autophagy process, thus canonical autophagy is considered as Beclin 1-dependent autophagy (which is the autophagy mode to be discussed in this chapter). To date, the cross talk between apoptosis and autophagy has been extensively investigated since both autophagy and apoptosis are evolutionarily conserved cellular mechanisms. The coregulation between apoptosis and autophagy may be formulated during evolution. It is potentially important in diverse aspects, such as developmental and tissue homeostasis, by conferring cells or organisms coordinating responses to stress signals.

Over the last decade, significant progress has been made to elucidate various molecular aspects in the interplay between autophagy and apoptosis, which can be classified into distinct paradigms based on: (i) the regulation of apoptosis by autophagy; (ii) autophagy regulation by apoptosis. Each of the regulations can be further subdivided into different mechanistic modes. In this chapter, we will focus on apoptosis-mediated negative regulation of autophagosome synthesis, and this will be discussed from the following perspectives: (i) the suppression of autophagosome synthesis by proteins with dual functions in autophagy and apoptosis; (ii) the blockage of autophagosome via protein inactivation by apoptosis-activating proteases.

APOPTOSIS INHIBITS AUTOPHAGOSOME BIOGENESIS VIA DUAL-FUNCTIONAL PROTEINS

Autophagosome Synthesis is Inhibited by Apoptosis Facilitator Bim

As an apoptosis facilitator protein, the BH3-only protein, Bim has capacity to inhibit Bcl-2 proteins and activate Bax/Bax proteins. In healthy cells, Bim is sequestered by dynein light chain 1 (LC8) to microtubules and remains inactive (Puthalakath et al., 1999). Recently, we found that Bim inhibits autophagy in addition to its pro-apoptotic function (Luo et al., 2012). This is the first identified molecule possessing dual effects in inhibiting autophagy and promoting apoptosis.

By biochemical assays, we unexpectedly found that Bim physically interacts with Beclin 1. With genetic knockdown of Bim by siRNA or shRNA, the levels in autophagosome synthesis markers, such as LC3-II levels and GFP-LC3 vesicle numbers, were remarkably increased, but the levels of autophagic substrates such as p62 significantly were decreased. Conversely, Bim overexpression inhibited the levels of LC3-II and the numbers of GFP-LC3, but increased the levels of autophagic substrate p62. These data suggest that Bim inhibits autophagosome formation. We confirmed the inhibitory effects of Bim on autophagy using Bim knockout mice (Luo et al., 2012). The effects of Bim on autophagy

are Beclin 1-dependent since Beclin 1 knockdown abolished the effects of Bim knockdown on autophagy. Interestingly, starvation dramatically reduced the Bim–Beclin 1 interaction by phosphorylating Bim at Theronine 116 (T116). With a series of mutations in Bim, we found that the strength of the Bim–Beclin 1 interaction coincided with that of the LC8–Bim interaction. Through *in vitro* binding and LC8 knockdown assays, we concluded that LC8 facilitates the Bim–Beclin 1 interaction and LC8–Bim–Beclin 1 forms a ternary complex. Phosphorylation of Bim at T116 disrupted the LC8–Bim interaction, thereby weakening the Bim–Beclin 1 interaction. These findings eventually led us to discover that the mechanism for autophagosome inhibition of Bim is due to Beclin 1 being mislocalized to microtubules from its autophagic site, ER, via its ternary interaction with Bim and LC8 (Luo et al., 2012; Luo and Rubinsztein, 2013). Given that Bim levels are often upregulated by apoptosis stimuli (Puthalakath et al., 2007), Bim-mediated apoptotic inhibition of autophagosome synthesis may represent an important paradigm in the aspect (Figure 4.3).

The effect of Bim in autophagy inhibition may be important for its critical roles in development and disease pathogenesis. Identifying the molecules like Bim with relevance to autophagy inhibition and apoptosis is critical to understand not only fundamental cellular processes but also disease pathogenesis, given that both autophagy and apoptosis are essential in regulating cellular processes and diseases.

Autophagosome Synthesis Is Inhibited by Apoptotic Diversion of Atg5/Atg12

Autophagosome formation requires ubiquitin-like conjugation of the autophagy proteins Atg12–Atg5 (Mizushima et al., 1998; Figure 4.2). Therefore, Atg5 and Atg12 are essential components of autophagosome core machinery. In addition to their roles in autophagy, Atg5 and Atg12 also have critical functions in inducing apoptosis upon diverse stress signals (Rubinstein et al., 2011; Yousefi et al., 2006). These suggest that the functions of Atg5 and Atg12 can be switched to apoptosis induction from autophagosome formation at death stimuli. By siRNA screening, Rubinstein et al (2011) discovered that Atg12 may mediate caspase activation in response to death stimuli. In contrast, knockdown of several other essential autophagy genes did not produce significant effects on apoptosis. This suggests that the role of Atg12 in apoptosis induction is independent of autophagosome formation or autophagy. It appears that free Atg12 rather than the Atg5–12 conjugate is capable of binding and inhibiting Bcl-2 anti-apoptotic proteins. This led to the finding that a BH3-like

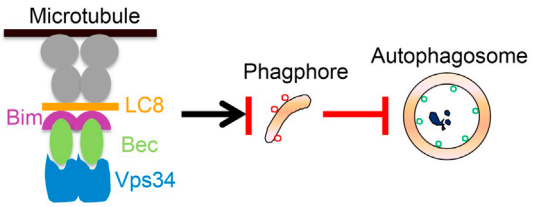

FIGURE 4.3 Apoptosis inhibits autophagosome formation via Bim. LC8 recruits Beclin 1 to the microtubule-based dynein motor complex via Bim (whose levels are often upregulated by apoptosis stimuli) and inhibits autophagosome formation by mislocalizing the Beclin 1–Vps34 complex to microtubules away from phagophores.

domain within Atg12 exerts its pro-apoptotic function by binding and inactivating Bcl-2 proteins in death stimulation conditions (Rubinstein et al., 2011). Therefore, apoptotic stimuli can compromise autophagosome synthesis by diverting free Atg12 to apoptotic machinery from the Atg5–12 conjugate, a component of autophagy core machinery. Interestingly, Atg5 is cleaved by calpain during apoptosis induction, and the cleaved Atg5 translocates to mitochondria to inactivate Bcl-xL's anti-apoptotic activity, thereby inducing mitochondrion-dependent apoptosis. With similar characteristic of Atg12 in apoptosis induction, unconjugated Atg5 rather than the Atg5–12 conjugate is involved in apoptosis process (Yousefi et al., 2006). These suggest that the roles of Atg5 in autophagy and apoptosis are reciprocally exclusive. Therefore, nonconjugated forms of Atg12 and Atg5, rather than conjugated Atg5–12, contribute to induction of apoptosis in death stimuli, and apoptosis induction in these cases inhibits autophagosome formation by switching the Atg5–12 conjugate that is essential for autophagosome formation, to nonconjugated Atg5 or Atg12. The unified molecular events suggest that apoptosis inhibition of autophagosome formation is a programmed cellular mechanism.

APOPTOSIS INHIBITION OF AUTOPHAGOSOME FORMATION BY PROTEASE ACTIVITY

Caspases play central roles in apoptosis, and they are also important in regulating autophagosome formation. The activation of caspases by apoptosis can inhibit autophagosome formation by proteolysis of the relevant proteins. To date, several autophagosome-regulatory proteins have been identified as targets for caspase-mediated cleavage, resulting in negative regulation of autophagosome formation. The caspase target proteins include Beclin 1, Atg3, Atg7, Atg9, Atg4D, and Ambra1 (Betin and Lane, 2009; Cho et al., 2009; Luo and Rubinsztein, 2010; Norman et al., 2010; Oral et al., 2012; Pagliarini et al., 2012). In addition, autophagosome-related proteins have also been reported to be cleaved by activated calpains (Yousefi et al., 2006).

Beclin 1–Vps34 Complex

Beclin 1 is the first autophagy core-machinery protein identified as a caspase substrate (Luo and Rubinsztein, 2010). Initially, we found that Bax-induced apoptosis markedly reduced autophagosome formation at the early stage of apoptosis, and subsequently revealed that caspase-mediated cleavage of Beclin 1 at D149 occurred when apoptosis is initiated. Interestingly, after the cleavage, both N- and C-terminal Beclin 1 fragments translocated into nuclei from cytoplasm where full-length Beclin 1 is located. Although wild-type Beclin 1 interacts with Vps34, the caspase-cleavage fragments of Beclin 1 did not. As such, the cleaved Beclin 1 no longer exerted autophagic activity as Beclin 1–Vps34 interaction is required for autophagosome biogenesis. Beclin 1 cleavage is a critical event whereby caspases inhibit autophagosome formation, as a noncleavable Beclin 1 mutant partially restored autophagy in cells overexpressing Bax. On the basis of our finding on caspase-mediated Beclin 1 cleavage, we further demonstrated that anti-apoptotic proteins, such as Bcl-2 proteins, restore autophagosome synthesis in the cells undergoing death stimuli (Luo

and Rubinsztein, 2010). These findings were subsequently confirmed by other independent studies (Li et al., 2011; Wirawan et al., 2010; Zhu et al., 2010). Interestingly, Wirawan et al (2010) later found that Beclin 1 is cleaved at D133 in addition to D149, and the C-terminal Beclin 1 fragment can translocate to mitochondria to induce mitochondrion-dependent apoptosis.

Activating Molecule in Beclin1-Regulated Autophagy (Ambra1), a WD40-containing E3 ligase, is a subunit of class III PI-3 kinase complex. Ambra1 is critical for Beclin 1-dependent autophagosome synthesis (Fimia et al., 2007). Upon apoptotic stimuli, a rapid reduction in Ambra1 levels was observed, and the reduction was reversed by inhibition of caspases and calpains. Caspases were shown to cleave Ambra1 at the D482 site, whereas calpains were involved in complete Ambra1 degradation (Pagliarini et al., 2012). Importantly, apoptotic stimuli further exacerbated cell viability via the inhibition of Ambra1-mediated autophagy, given that autophagy alleviates apoptotic insults. On the other hand, Ambra1 overexpression counteracted apoptosis by restoring autophagy. A caspase noncleavable Ambra1 mutant restored autophagy and exerted anti-apoptotic effect more efficiently (Pagliarini et al., 2012). Therefore, Ambra1 is an important target of apoptotic proteases to block autophagosome biogenesis.

Other Autophagy Component Cleavage by Caspases

In addition to Beclin 1–Vps34 complex components, other core autophagy machinery proteins are also cleaved after apoptotic stimulation (Norman et al., 2010). These cleavages are supposed to also negatively regulate Beclin 1-dependent autophagosome formation, given that the components are also required for the process. Atg3 cleavage occurred with cell death induced by tumor necrosis factor α (TNFα) and tumor necrosis factor-related apoptosis inducing ligand (TRAIL), and caspase-8 overexpression led to Atg3 degradation. However, blockage of caspases or overexpression of a noncleavable Atg3 protein restored autophagic activity upon death receptor stimulation. Therefore, Atg3 is a potential link between apoptosis and autophagy during receptor-activated cell death (Oral et al., 2012). Moreover, Atg9, Atg7, and Atg4 homologs can also be cleaved by caspase-3 (Norman et al., 2010). Interestingly, caspase-3-mediated Atg4D cleavage appears to lead to its activation in LC3 lipidation (Betin and Lane, 2009).

Apoptosis-Induced Calpain Cleavage of Autophagy Components

Besides caspase-mediated cleavage in the components for autophagosome synthesis, apoptosis-induced calpain activation also plays a role in negative regulation of autophagosome formation. Atg5 is the first autophagy protein reported to be cleaved by calpains when apoptosis occurs. The resulting N-terminal fragment of Atg5 translocates to the mitochondrion by an unknown mechanism, where it mediates the release of cytochrome c by interacting with the Bcl-2-like protein Bcl-xL (Yousefi et al., 2006). Calpains are also involved in Ambra1 cleavage, and they cooperate with caspases for complete degradation of Ambra1 (Pagliarini et al., 2012). In addition, Norman et al. reported that the majority of human Atg (hAtg) proteins can be cleaved by calpain 1, which is activated in some apoptotic events and other death modes (Norman et al., 2010). Therefore, apoptosis can exert inhibitory effects on

FIGURE 4.4 The inhibitory effects of apoptosis on autophagosome synthesis by proteolysis of autophagy factors. The autophagy core machinery proteins (Atgs) are cleaved by apoptosis-activated proteases, leading to autophagy inhibition.

autophagosome formation and autophagy through proteolysis of multiple autophagy components by both caspases and calpains (Figure 4.4).

DISCUSSION

Autophagy is generally believed to be a pro-survival factor as it is required for cellular energy and nutrition homeostasis, and removing toxic intracellular components (Moreau et al., 2010). When cells undergo stress stimuli, pro-survival and pro-death pathways are simultaneously initiated. The fate of the cells is determined by the complex balance between these pathways. The interaction between autophagy and apoptosis may be closely implicated in cell death and survival. Apoptosis inhibition in autophagosome formation may exert an important effect in the survival–death balance.

Accumulating evidence suggests that autophagy inactivation by apoptotic machinery is a crucial event for cell death execution (Rubinstein and Kimchi, 2012). A plausible explanation for this phenomenon is that inhibition of autophagy by death stimuli, prior to apoptosis or at the early stage of apoptosis, minimizes simultaneous activation events in cellular pro-survival and pro-death, thereby enabling the dying cells to more rapidly reach the "point of no return." For example, in the early stage of apoptosis, autophagy is suppressed by caspase-mediated Beclin 1 cleavage, and the pro-survival function of autophagy is blocked under these conditions. The action in autophagy inhibition, therefore, confers positive feedback loop mechanism to apoptosis initiation (Luo and Rubinsztein, 2010; Wirawan et al., 2010). This is also the case in other examples of caspase cleavage of autophagic proteins. Pagliarini and colleagues reported that expression of a caspase-resistant mutant form of the autophagy protein Ambra1 gives rise to partial protection from apoptotic cell death, which is associated with higher levels of autophagy (Pagliarini et al., 2012). Therefore, apoptosis inhibition in autophagosome formation and autophagy confers the cells to be dismantled more rapidly. The quick cell death event is critical as it allows organisms to clear up the dead cells in timely manner by macrophage uptake to avoid further immune responses. Regulation of autophagosome synthesis by apoptosis depends on caspase activation that can be blocked by genetic inhibition or pharmacological inhibitors of caspases, such as z-VAD-fmk. Therefore, caspase inhibition can have dual effects in both apoptosis and autophagy. Anti-apoptotic proteins Bcl-2 is well-known to suppress autophagy by physically interacting with Beclin 1 (Pattingre et al., 2005). The function of Bcl-2 is important to maintain basal autophagy in cells at lower levels in normal conditions. However, Bcl-2 proteins can reverse autophagy reduction in the cells subject to apoptotic stimuli through caspase inhibition and anti-apoptotic function (Luo and Rubinsztein, 2010). Therefore, depending on cellular contexts, Bcl-2 proteins may exert opposite effects in autophagy inhibition and autophagy protection.

On the other hand, caspases could paradoxically activate autophagy by an unidentified mechanism. Caspase activation occurs prior to the appearance of autophagosomes during steroid-activated apoptosis of *Drosophila* salivary glands (Lee and Baehrecke, 2001), Surprisingly, starvation-induced autophagy in *Drosophila* nurse cells was shown to require the caspase Dcp1 (Hou et al., 2008; Kim et al., 2010). Given that apoptosis did not happen with the caspase activation in these studies, caspases required for autophagosome formation in these conditions appear apoptosis-insufficient. These suggest that apoptosis-sufficient caspase activation is required for the blockage of autophagosome synthesis.

Clarifying the interaction between apoptosis and inhibition of autophagosome synthesis is important as both apoptosis and autophagy are critical for the pathogenesis of various diseases. The cross talk between autophagy and apoptosis has been extensively reported in heart diseases (Nishida et al., 2008) and is involved in trauma and sepsis (Hsieh et al., 2009). In neurodegenerative diseases, apoptosis and autophagy degeneration generally occurs simultaneously (Anglade et al., 1997), and Beclin 1 cleavage was observed in Alzheimer's Disease brain (Rohn et al., 2011). Further studies on the mechanisms to apoptotic inhibition of autophagosome synthesis may lead to better understanding to the pathogenesis of the diseases and could provide insight into potential therapeutic treatment of the diseases.

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References

Anglade, P., Vyas, S., Javoy-Agid, F., et al., 1997. Apoptosis and autophagy in nigral neurons of patients with Parkinson's disease. Histol. Histopathol. 12, 25–31.

Barnhart, B.C., Alappat, E.C., Peter, M.E., 2003. The CD95 type I/type II model. Semin. Immunol. 15, 185–193.

Bender, C.E., Fitzgerald, P., Tait, S.W., et al., 2012. Mitochondrial pathway of apoptosis is ancestral in metazoans. Proc. Natl. Acad. Sci. U.S.A. 109, 4904–4909.

Betin, V.M., Lane, J.D., 2009. Atg4D at the interface between autophagy and apoptosis. Autophagy 5, 1057–1059.

Cho, D.H., Jo, Y.K., Hwang, J.J., et al., 2009. Caspase-mediated cleavage of ATG6/Beclin-1 links apoptosis to autophagy in HeLa cells. Cancer Lett. 274, 95–100.

Fimia, G.M., Stoykova, A., Romagnoli, A., et al., 2007. Ambra1 regulates autophagy and development of the nervous system. Nature 447, 1121–1125.

Hanada, T., Noda, N.N., Satomi, Y., et al., 2007. The Atg12-Atg5 conjugate has a novel E3-like activity for protein lipidation in autophagy. J. Biol. Chem. 282, 37298–37302.

Hosokawa, N., Hara, T., Kaizuka, T., et al., 2009. Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. Mol. Biol. Cell 20, 1981–1991.

Hou, Y.C., Chittaranjan, S., Barbosa, S.G., et al., 2008. Effector caspase Dcp-1 and IAP protein Bruce regulate starvation-induced autophagy during *Drosophila melanogaster* oogenesis. J. Cell Biol. 182, 1127–1139.

Hsieh, Y.C., Athar, M., Chaudry, I.H., 2009. When apoptosis meets autophagy: deciding cell fate after trauma and sepsis. Trends Mol. Med. 15, 129–138.

Itakura, E., Kishi, C., Inoue, K., et al., 2008. Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. Mol. Biol. Cell 19, 5360–5372.

Kaufmann, A., Beier, V., Franquelim, H.G., et al., 2014. Molecular mechanism of autophagic membrane-scaffold assembly and disassembly. Cell 156, 469–481.

Kim, J., Kundu, M., Viollet, B., et al., 2011. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat. Cell Biol. 13, 132–141.

Kim, Y.I., Ryu, T., Lee, J., et al., 2010. A genetic screen for modifiers of *Drosophila* caspase Dcp-1 reveals caspase involvement in autophagy and novel caspase-related genes. BMC Cell Biol. 11, 9.
Lee, C.Y., Baehrecke, E.H., 2001. Steroid regulation of autophagic programmed cell death during development. Development 128, 1443–1455.
Levine, B., Kroemer, G., 2008. Autophagy in the pathogenesis of disease. Cell 132, 27–42.
Li, H., Wang, P., Sun, Q., et al., 2011. Following cytochrome *c* release, autophagy is inhibited during chemotherapy-induced apoptosis by caspase 8-mediated cleavage of Beclin 1. Cancer Res. 71, 3625–3634.
Lum, J.J., DeBerardinis, R.J., Thompson, C.B., 2005. Autophagy in metazoans: cell survival in the land of plenty. Nat. Rev. Mol. Cell Biol. 6, 439–448.
Luo, S., Rubinsztein, D.C., 2007. Atg5 and Bcl-2 provide novel insights into the interplay between apoptosis and autophagy. Cell Death Differ. 14, 1247–1250.
Luo, S., Rubinsztein, D.C., 2010. Apoptosis blocks Beclin 1-dependent autophagosome synthesis: an effect rescued by Bcl-xL. Cell Death Differ. 17, 268–277.
Luo, S., Rubinsztein, D.C., 2013. BCL2L11/BIM: a novel molecular link between autophagy and apoptosis. Autophagy 9, 104–105.
Luo, S., Garcia-Arencibia, M., Zhao, R., et al., 2012. Bim inhibits autophagy by recruiting Beclin 1 to microtubules. Mol. Cell 47, 359–370.
Mizushima, N., Noda, T., Yoshimori, T., et al., 1998. A protein conjugation system essential for autophagy. Nature 395, 395–398.
Moreau, K., Luo, S., Rubinsztein, D.C., 2010. Cytoprotective roles for autophagy. Curr. Opin. Cell Biol. 22, 206–211.
Nishida, K., Yamaguchi, O., Otsu, K., 2008. Crosstalk between autophagy and apoptosis in heart disease. Circ. Res. 103, 343–351.
Noda, N.N., Fujioka, Y., Hanada, T., et al., 2013. Structure of the Atg12-Atg5 conjugate reveals a platform for stimulating Atg8-PE conjugation. EMBO Rep. 14, 206–211.
Norman, J.M., Cohen, G.M., Bampton, E.T., 2010. The *in vitro* cleavage of the hAtg proteins by cell death proteases. Autophagy 6, 1042–1056.
Oral, O., Oz-Arslan, D., Itah, Z., et al., 2012. Cleavage of Atg3 protein by caspase-8 regulates autophagy during receptor-activated cell death. Apoptosis 17, 810–820.
Otomo, C., Metlagel, Z., Takaesu, G., et al., 2013. Structure of the human ATG12~ATG5 conjugate required for LC3 lipidation in autophagy. Nat. Struct. Mol. Biol. 20, 59–66.
Pagliarini, V., Wirawan, E., Romagnoli, A., et al., 2012. Proteolysis of Ambra1 during apoptosis has a role in the inhibition of the autophagic pro-survival response. Cell Death Differ. 19, 1495–1504.
Pattingre, S., Tassa, A., Qu, X., et al., 2005. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell 122, 927–939.
Puthalakath, H., Huang, D.C., O'Reilly, L.A., et al., 1999. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. Mol. Cell 3, 287–296.
Puthalakath, H., O'Reilly, L.A., Gunn, P., et al., 2007. ER stress triggers apoptosis by activating BH3-only protein Bim. Cell 129, 1337–1349.
Riedl, S.J., Salvesen, G.S., 2007. The apoptosome: signalling platform of cell death. Nat. Rev. Mol. Cell Biol. 8, 405–413.
Rohn, T.T., Wirawan, E., Brown, R.J., et al., 2011. Depletion of Beclin-1 due to proteolytic cleavage by caspases in the Alzheimer's disease brain. Neurobiol. Dis. 43, 68–78.
Romanov, J., Walczak, M., Ibiricu, I., et al., 2012. Mechanism and functions of membrane binding by the Atg5-Atg12/Atg16 complex during autophagosome formation. Embo J. 31, 4304–4317.
Rubinstein, A.D., Kimchi, A., 2012. Life in the balance—a mechanistic view of the crosstalk between autophagy and apoptosis. J. Cell Sci. 125, 5259–5268.
Rubinstein, A.D., Eisenstein, M., Ber, Y., et al., 2011. The autophagy protein Atg12 associates with antiapoptotic Bcl-2 family members to promote mitochondrial apoptosis. Mol. Cell 44, 698–709.
Shamas-Din, A., Brahmbhatt, H., Leber, B., et al., 2011. BH3-only proteins: orchestrators of apoptosis. Biochim. Biophys. Acta 1813, 508–520.
Suzuki, K., Kirisako, T., Kamada, Y., et al., 2001. The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. Embo J. 20, 5971–5981.
Tait, S.W., Green, D.R., 2010. Mitochondria and cell death: outer membrane permeabilization and beyond. Nat. Rev. Mol. Cell Biol. 11, 621–632.

Vercammen, D., Declercq, W., Vandenabeele, P., et al., 2007. Are metacaspases caspases? J. Cell Biol. 179, 375–380.
Wirawan, E., Vande Walle, L., Kersse, K., et al., 2010. Caspase-mediated cleavage of Beclin-1 inactivates Beclin-1-induced autophagy and enhances apoptosis by promoting the release of proapoptotic factors from mitochondria. Cell Death Dis. 1, e18.
Youle, R.J., Strasser, A., 2008. The BCL-2 protein family: opposing activities that mediate cell death. Nat. Rev. Mol. Cell Biol. 9, 47–59.
Yousefi, S., Perozzo, R., Schmid, I., et al., 2006. Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. Nat. Cell Biol. 8, 1124–1132.
Zhu, Y., Zhao, L., Liu, L., et al., 2010. Beclin 1 cleavage by caspase-3 inactivates autophagy and promotes apoptosis. Protein Cell 1, 468–477.

CHAPTER

5

Is Selective Autophagy Distinct from Starvation-Induced Autophagy?

Gérald Guillebaud and Marc Germain

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Abstract

Macroautophagy (autophagy) is a catabolic process required to deliver cytoplasmic content to lysosomes for degradation. During nutrient starvation, cells activate autophagy to promote nutrient recycling and survival. In addition, a selective form of autophagy is required to degrade large intracellular aggregates and dysfunctional organelles, the failure of which is linked to neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and Huntington's disease. In fact, pharmacological induction of autophagy in genetic models of neurodegenerative diseases improves aggregate clearance and neurological outcomes, providing a proof of concept for the use of autophagy inducers for the treatment of these diseases. However, while selective autophagy has a clear neuroprotective role, starvation-induced autophagy is detrimental to neurons under some circumstances. This suggests that these two forms of autophagy are at least partially distinct and that by understanding the mechanisms regulating them, it will be possible to identify autophagy inducers specific for selective autophagy.

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INTRODUCTION

One of the key features of neurodegenerative diseases is the accumulation of damaged cellular components such as dysfunctional mitochondria or ubiquitinated protein aggregates (Frake et al., 2015; Rogov et al., 2014). In healthy cells, this material is degraded through a process called macroautophagy (thereafter autophagy) that is characterized by the formation of a double-membrane vesicle, the autophagosome, which delivers intracellular material to lysosomes for degradation (Mehrpour et al., 2010; Mizushima and Komatsu, 2011; Rogov et al., 2014). Importantly, autophagy is altered in neurodegenerative diseases and pharmacological activation of autophagy has been proposed as a therapeutic avenue to decrease aggregate formation and promote neuronal survival (Frake et al., 2015; Hochfeld et al., 2013).

Autophagy is induced by nutrient deprivation to promote nutrient recycling and cellular survival during starvation (Mehrpour et al., 2010; Mizushima and Komatsu, 2011). However, it is the basal level of autophagy present in all cells that is responsible for the degradation of damaged organelles and large protein aggregates, a form of autophagy referred to as quality control or selective autophagy (Mizushima and Komatsu, 2011; Rogov et al., 2014). Importantly, these roles of macroautophagy (starvation, quality control) are required in distinct biological contexts. For example, while starvation-induced autophagy promotes survival of cancer cells, it is the selective form of autophagy that plays a survival role in neurons by preventing the accumulation of potentially toxic damaged cellular components. In fact, some evidence suggests that starvation-induced autophagy can be detrimental to neurons, raising the possibility that selective autophagy and starvation-induced autophagy are distinct forms of autophagy. In this review, we will address this question by first providing an overview of the core autophagy machinery and the pathways regulating it. We will then address the mechanisms regulating selective autophagy and their relationship with starvation-induced autophagy.

OVERVIEW OF AUTOPHAGY

During autophagy, intracellular material is packaged into an autophagosome to be delivered to lysosomes for degradation (Figure 5.1). In the case of starvation-induced autophagy, the cargo is thought to consist of random cytoplasmic material that will be degraded to maintain the pool of intracellular nutrients. In contrast, selective autophagy requires the recognition of specific cargo by autophagy receptors and adaptors that will stimulate its inclusion within an autophagosome (Mehrpour et al., 2010; Mizushima and Komatsu, 2011; Rogov et al., 2014). Despite these differences, the core autophagy machinery is the same for both types of autophagy and requires the action of a set of highly conserved proteins.

The process of autophagy is divided in several steps that go from the nucleation of the initial sequestering organelle, the phagophore, to the delivery of the completed autophagosome to lysosomes. In mammals, autophagy initiation is controlled by the kinase ULK1 and its closely related homologue ULK2, which are constitutively associated with the scaffolding protein FIP200 as well as ATG13 and ATG101 (Abada and Elazar, 2014; Alers et al., 2012). Under nutrient-replete conditions, mTOR keeps ULK1 inactive by phosphorylating both ULK1 and ATG13. mTOR inactivation during starvation leads to the rapid activation

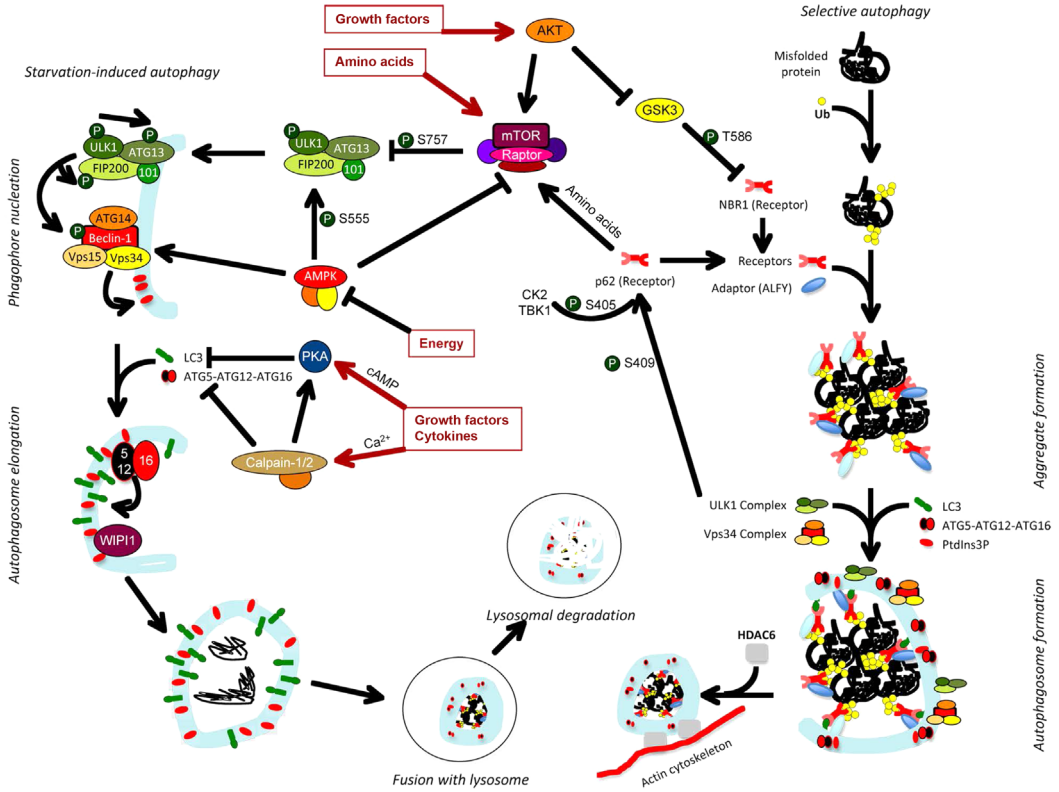

FIGURE 5.1 Regulation of starvation-induced autophagy and selective autophagy. Starvation-induced autophagy is prevented by growth factors and cytokines through the activation of mTOR and PKA. mTOR inhibits the activation of the autophagy-initiating complex (ULK1 complex). Upon mTOR inhibition, ULK1 is activated and recruited to a phagophore initiation site where it initiates autophagosome formation by activating the Vps34 complex. In contrast, selective autophagy is initiated when (usually) ubiquitinated substrate is recognized by autophagy receptors such as p62 or NBR1, which in turn recruits the autophagy machinery. Importantly, the recruitment of both p62 and NBR1 to protein aggregates is regulated by phosphorylation.

of ULK1 and its recruitment to intracellular membranes, the nature of which is not fully resolved but probably includes sites on the endoplasmic reticulum (Bernard and Klionsky, 2013). There, the ULK1 complex activates phagophore nucleation through the recruitment and activation of a Class III PI3 kinase complex (Vps34 complex), which comprises Vps34 (PI3 kinase) as well as the regulatory proteins Beclin-1, ATG14, and Vps15. Vps34-dependent generation of phosphatidylinositol-3-phosphate (PtdIns3P) at the nucleation site causes the recruitment of the machinery required for autophagosome elongation and closure (Alers et al., 2012; Itakura and Mizushima, 2010). This includes PtdIns3P-binding proteins such as WIPI1 and two ubiquitin-like conjugation systems: ATG5-ATG12 and LC3 (Alers et al., 2012; Itakura and Mizushima, 2010; Mizushima and Komatsu, 2011). The conjugation of ATG5 and ATG12 and their association with ATG16 occurs in a constitutive

manner. The ATG5 complex is then selectively recruited to the initiation site where it catalyzes the conjugation of LC3 to phosphatidyletanolamine (PE), an event required for autophagosome elongation and closure. Once completed, the autophagosome fuses with a lysosome through a process that requires components of the endocytic pathway (Mehrpour et al., 2010; Mizushima and Komatsu, 2011).

The best understood pathway leading to starvation-induced autophagosome formation is regulated by mTOR, which directly inhibits autophagy by phosphorylating and inactivating the ULK1 complex (Alers et al., 2012; Mizushima and Komatsu, 2011). mTOR is a central hub for growth regulation as it integrates important intracellular and extracellular cues such as nutrient availability and the presence of growth factors to regulate protein translation and cell growth (Bove et al., 2011; Laplante and Sabatini, 2012). Growth factors stimulate mTOR activation through AKT-dependent inhibition of Tsc1/Tsc2, while amino acids activate mTOR through its RAG-dependent recruitment to lysosomes (Jewell et al., 2013). Therefore, either loss of growth factor signaling or decreased amino acid content inhibits mTOR and activates autophagy.

In addition to these signals, mTOR is regulated by ATP levels. Decreased ATP levels (and concomitant increase in AMP) activate AMP-dependent protein kinase (AMPK). AMPK then acts at several steps in the mTOR pathway to inhibit its function and activate autophagy (Hardie, 2014; Kim et al., 2013; Mihaylova and Shaw, 2011). First, AMPK inhibits mTOR by two mechanisms: the activation of Tsc1/Tsc2, a mTOR inhibitory complex, and through the direct phosphorylation and inhibition of mTOR (Hardie, 2014; Mihaylova and Shaw, 2011). Second, AMPK directly activates autophagy by phosphorylating ULK1 and the Vps34 complex component Beclin-1 (Hardie, 2014; Kim et al., 2013; Mihaylova and Shaw, 2011). Importantly, growth factors and cytokines can also inhibit the Vps34 complex in an mTOR-independent fashion (Lipinski et al., 2010). The pathways involved are not well characterized but possibly include the activation of the IP3/Ca^{2+}/Calpain pathway and cAMP/PKA (Vakifahmetoglu-Norberg et al., 2015; Williams et al., 2008; Zhang et al., 2007). For example, yeast PKA phosphorylates and inhibits ATG13 while in mammals, PKA has been shown to inhibit autophagy by phosphorylating LC3 (McEwan and Dikic, 2011). The regulation of autophagy is summarized in Figure 5.1.

SELECTIVE AUTOPHAGY

Autophagosomes formed during starvation are thought to include nonspecific cytoplasmic cargo that will be degraded in lysosomes to provide the nutrient required for cell survival (Mehrpour et al., 2010; Mizushima and Komatsu, 2011). In contrast, during selective autophagy, autophagosomes are specifically generated around the cargo to be degraded through recognition by autophagy receptors and adaptors. These in turn recruit the autophagy machinery to the nascent autophagosome (Birgisdottir et al., 2013; Isakson et al., 2013; Rogov et al., 2014). Several such receptors/adaptors have been identified in recent years and shown to promote degradation of specific cargo ranging from ubiquitinated proteins to intracellular pathogens (Isakson et al., 2013). Compared to the starvation-induced pathway described above, selective autophagy therefore follows an outside-out mechanism for autophagosome formation (Figure 5.1).

During selective autophagy, the cargo is first modified (generally ubiquitinated) and recognized by autophagic receptors. One of the most studied receptors is p62, a multidomain protein that directly binds to ubiquitin through a UBA domain but also interacts with the autophagosome protein LC3 (Birgisdottir et al., 2013; Lin et al., 2013). p62 directly promotes autophagosome formation around the cargo by recruiting other proteins such as the autophagy adaptor Alfy, which in turn recruit the core autophagy machinery (ULK1 complex, Vps34 complex, ATG5/12/16, LC3) (Birgisdottir et al., 2013; Lin et al., 2013; Yamamoto and Simonsen, 2011). Initial phagophore membranes could also be recruited to the aggregation site before ULK1 and Vps34. This is based on the observation that in yeast, ATG8 (LC3 homologue) conjugation occurs constantly and independently of autophagy induction (Nair et al., 2012; Nakatogawa et al., 2012; Yu et al., 2012). It was thus proposed that cargo-bound autophagy receptors and adaptors recruit these transient phagophores to initiate autophagosome formation (Rogov et al., 2014), leading to the recruitment of the ULK1 and Vps34 complexes to trigger autophagosome formation.

This model raises important questions concerning the regulation of selective autophagy. First, are there signals promoting phagophore formation to accommodate an increase in selective autophagy substrates to be degraded? Otherwise, it would imply the presence of a set limit to the capacity of the system under nutrient-replete conditions. It is also unclear how ULK1 and Vps34 are activated upon their recruitment under these conditions. Given the links between autophagy and disease described below, these are critical questions that are only starting to be addressed.

AUTOPHAGY AND DISEASE

Accumulation of protein aggregates is a common feature of neurodegenerative diseases. In fact, decreased autophagic clearance has been observed in several neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), and Huntington's disease (HD) (Jimenez-Sanchez et al., 2012; Lynch-Day et al., 2012). Consistent with this, mutations in genes linked to PD (α-Synuclein, LRRK2, PINK1, Parkin) and HD (Htt) lead to impaired autophagy (Gomez-Suaga et al., 2012; Heng et al., 2010; Martinez-Vicente et al., 2010; Narendra et al., 2010a,,b; Winslow et al., 2010). These observations, together with the accumulation of aggregated material in neurodegenerative diseases, led to the suggestion that autophagy can be used as a therapeutic approach for these diseases. Indeed, as described below, induction of autophagy can reduce the accumulation of protein aggregates caused by proteins linked to neurodegeneration (α-Synuclein, Htt, Aβ,) and improve behavior in animal models (Majumder et al., 2011; Ozcelik et al., 2013; Pickford et al., 2008; Proenca et al., 2013; Ravikumar et al., 2004; Shibata et al., 2006; Spilman et al., 2010).

Pharmacological Induction of Autophagy in Neurodegenerative Diseases

Two sets of experiments originally demonstrated a role for autophagy in the accumulation of protein aggregates and neurodegeneration. First, deletion of essential autophagy genes (ATG5, ATG7) in the brain has been shown to lead to the accumulation of ubiquitinated protein aggregates and neurodegeneration (Hara et al., 2006; Komatsu et al., 2006).

Around the same time, the laboratory of Dr David Rubinsztein demonstrated that several aggregate prone proteins involved in neurodegenerative diseases are degraded by autophagy and that increasing autophagy promotes their clearance (Ravikumar et al., 2002, 2004; Webb et al., 2003). This was followed by a large number of studies aimed at pharmacologically inducing autophagy to improve the neurological outcome in various models of neurodegenerative diseases. Importantly, several of these studies demonstrated that *in vivo* modulation of autophagy improves behavior in genetic models of AD, HD, and PD, providing the proof of concept for the use of autophagy activators to treat neurodegenerative diseases (Caccamo et al., 2010, 2014; Menzies et al., 2015; Pickford et al., 2008; Ravikumar et al., 2004; Rose et al., 2010; Spencer et al., 2009). This has then led to the identification of new potential autophagy activators from screens of libraries of drugs already in clinical use (Levine et al., 2015; Lipinski et al., 2010; Williams et al., 2008; Zhang et al., 2007).

Most of the studies manipulating autophagy to improve the outcome of neurodegenerative diseases have targeted one of two signaling pathways linked to autophagy: mTOR and IP3/Ca^{2+}/Calpain. A large proportion of the original studies have used mTOR inhibitors such as rapamycin to increase autophagy (Berger et al., 2006; Crews et al., 2010; Majumder et al., 2011; Menzies et al., 2010; Ozcelik et al., 2013; Pandey et al., 2007; Ravikumar et al., 2002, 2004; Spilman et al., 2010; Wang et al., 2009; Webb et al., 2003). While already in clinical use and efficient at clearing aggregates in cells and animal models, these compounds raise some issues for their long-term use in the context of neurodegeneration (Hochfeld et al., 2013). First, mTOR regulates several cellular processes in addition to autophagy, increasing the risks of important side effects during long-term treatments. Second, rapamycin is an immunosuppressant, raising further issues for its use as a treatment for neurodegenerative diseases.

To get around these issues, strategies to increase autophagy in an mTOR-independent fashion have been developed and used successfully to reduce aggregate load in various models of neurodegeneration (Levine et al., 2015; Vakifahmetoglu-Norberg et al., 2015). Several of these compounds act through an IP3/Ca^{2+}/Calpain pathway. For example, inhibition of L-Type Ca^{2+} channels or the IP3 pathway promotes degradation of mutant Huntingtin protein and improves motor symptoms in a HD mouse model (Rose et al., 2010; Williams et al., 2008; Zhang et al., 2007). A key effector downstream of these Ca^{2+} signals is the Ca^{2+}-dependent proteases Calpain-1/2. These Calpains inhibit autophagy directly by cleaving and inactivating ATG5 (Xia et al., 2010), and indirectly by activating the Gαs subunit of heterotrimeric G proteins, leading to increased cAMP and PKA activity (Williams et al., 2008). The role of Calpains in the regulation of autophagy-dependent aggregate clearance is further supported by a recent study indicating that Calpain inhibition *in vivo* in *Drosophila* and in mice decreased the accumulation of mutant Htt and improved motor behavior (Menzies et al., 2015).

The above studies support the idea that modulating autophagy could provide an efficient way to clear aggregates and improve cognitive and motor capacities of patients with neurodegenerative diseases. While this is highly promising, some key issues remain. First, the fact that pharmacological autophagy inducers target pathways that have important roles in addition to autophagy regulation remains an issue for chronic treatment of neurodegenerative diseases. More importantly, selective autophagy and starvation-induced autophagy have opposite roles under some circumstances.

For example, autophagy is detrimental to neurons under conditions associated with starvation-induced autophagy, such as following a stroke (Koike et al., 2008; Wen et al., 2008; Xing et al., 2012; Zhang et al., 2013). During a stroke, blood flow to the affected area is interrupted, leading to decreased oxygen and glucose supply to neurons and neuronal death. However, reestablishment of nutrient supply (reperfusion) also leads to neuronal damage, albeit through different mechanisms (Li et al., 1995; Siesjo et al., 1995). Importantly, a recent study (Zhang et al., 2013) indicated that while inhibiting autophagy during ischemia protects in against cell death in several stroke models, its inhibition during reperfusion is detrimental. This is because activation of selective autophagy is required during reperfusion to clear damaged mitochondria to avoid further injury (Zhang et al., 2013).

A similar dichotomy is observed in cancer. In the context of tumor formation, selective autophagy has been suggested to act as a tumor suppressor by decreasing cellular damage and thus the chance of tumorigenic mutations, although this remains controversial (White, 2015). On the other hand, as starvation-induced autophagy is required for cell survival in the hypoxic, low nutrient environment of the tumor, oncogenic potential of autophagy is well established (White, 2015). As with the role of autophagy in neurons, this illustrates the opposite roles that starvation-induced and selective autophagy can play in a pathological context. In fact, recent reports suggest that while these two forms of autophagy share the machinery to generate autophagosomes, they are regulated in a distinct fashion (Lee et al., 2010; Suzuki et al., 2014; Zhang et al., 2013). It is therefore crucial to better define the mechanisms regulating selective and starvation-induced autophagy to better target aggregate degradation as a therapeutic target of neurodegenerative diseases.

Are Selective Autophagy and Starvation-Induced Autophagy Two Distinct Forms of Autophagy?

The first evidence of a mechanistic distinction between selective and starvation-induced autophagy came from studying HDAC6, a cytoplasmic histone deacetylase that regulates cytoskeleton dynamics (Lee et al., 2010). Under fed conditions, HDAC6 deletion in mouse embryonic fibroblasts (MEFs) prevents the fusion of autophagosomes with lysosomes and impairs degradation of aggregated proteins. Importantly, lysosomal fusion is completely rescued during starvation, indicating that the two processes are regulated in a distinct fashion. The authors further demonstrated a specific role of the actin cytoskeleton in the fusion of selective but not starvation-induced autophagosomes with lysosomes, providing an important insight into the differences between these two forms of autophagy (Lee et al., 2010). The distinction between these two forms of autophagy is not limited to autophagosome fusion but also extend to autophagosome formation. For example, the autophagy adaptor Alfy is required for autophagosome biogenesis during selective autophagy but impairs starvation-induced autophagy (Filimonenko et al., 2007; Yamamoto and Simonsen, 2011). Similarly, disruption of the interaction between ATG13 and LC3 impairs the recruitment of LC3 to autophagosomes predominantly under fed conditions (Suzuki et al., 2014).

In contrast to selective autophagosomes, starvation-induced autophagosomes are thought to contain random cytoplasmic material (Rogov et al., 2014). It therefore remains unclear how mTOR inhibition (starvation pathway) can stimulate the formation of selective autophagosomes. In fact, as downregulation of the autophagy adaptor Alfy promotes

the degradation of random material at the expense of protein aggregates during starvation (Yamamoto and Simonsen, 2011), it is possible that starvation-induced autophagosomes compete with selective autophagosomes under some circumstances by promoting nucleation at sites devoid of specific cargo. While this is an intriguing possibility, the fact that mTOR inhibitors promote aggregate degradation in an autophagy-dependent manner (Berger et al., 2006; Menzies et al., 2010; Pandey et al., 2007) indicates that some mechanisms exist to link starvation-induced and selective autophagosome formation. Nevertheless, the studies discussed above suggest that selective autophagy and starvation-induced autophagy are at least partially distinct and that therefore, a more precise targeting of selective autophagy could represent a promising therapeutic target for the treatment of neurodegenerative diseases.

As stated above, the regulation of the two main autophagy initiation complexes, ULK1 and Vps34, remains poorly understood in the context of selective autophagy, especially in the context of the inside-out model of selective autophagosome formation where the activation of these complexes is not the initiating event. In fact, two possibilities arise from this model: either Vps34 and ULK1 can be activated in an mTOR-independent fashion or there is always a low, constitutive mTOR resistant pool of active ULK1 to maintain the formation of selective autophagosomes. The latter would suggest that the selective autophagy pathway can be saturated and therefore explain the progressive accumulation of protein aggregates occurring in neurons during aging and neurodegenerative diseases. It is however more likely that mechanisms exist to regulate selective autophagy flux. In that context, the identification of selective autophagy-specific ULK1 or Beclin-1 kinases would provide important insights concerning the regulation of starvation-induced autophagy. The stress-induced kinase JNK could provide such a signaling mechanism, albeit in an indirect fashion. The anti-apoptotic BCL-2 homologue BCL-2 inhibits Beclin-1 by binding to its BH3 domain and this interaction is disrupted by JNK-dependent phosphorylation of BCL-2, leading to the induction of autophagy (Wei et al., 2008). Interestingly, JNK has been shown to be activated in neurodegenerative diseases (Mehan et al., 2011), suggesting that it can be activated by aggregate formation. A second way to disrupt the interaction between Beclin-1 and BCL-2 is through the induction of BH3-only BCL-2 homologues, which displace the BH3 domain of Beclin-1 from the binding pocket in BCL-2 (Maiuri et al., 2007). As BH3-only proteins are induced by stress (Lomonosova and Chinnadurai, 2008), it is therefore possible that they regulate Beclin-1 activation during selective autophagy, although this remains speculative.

In addition to the activation of the Vps34 complex by Beclin-1/BCL-2 interaction, recent studies suggest a more direct control of selective autophagosome formation whereby local mTOR inhibition could drive ULK1 activation at aggregation sites. This is based on the observation that mTOR associates with Htt-positive aggregates where it gets inactivated (Ravikumar et al., 2004) while the autophagy receptor p62 interacts with mTOR and promotes its activation on lysosomes (Duran et al., 2011). p62 is present in three distinct locations within a cell: soluble in the cytosol, in foci associated with aggregated material, and on lysosomal membranes. p62 is required at this latter location to activate mTOR in responses to amino acids, but not extracellular signals (PI3K–AKT pathway) (Duran et al., 2011). The presence of protein aggregates could therefore favor the recruitment of p62 at aggregation sites at the expense of lysosomes, thereby sequestering and inactivating mTOR. As

the ULK1 complex physically interacts with mTOR (Alers et al., 2012), this would in turn lead to ULK1 activation at aggregates. This model is also supported by the observation that p62 phosphorylation at several sites promotes its recruitment to aggregates (Ichimura et al., 2013; Lim et al., 2015; Ro et al., 2014). In particular, the phosphorylation of p62 on S409 by ULK1 during selective autophagy, but not starvation-induced autophagy, is required for the clearance of protein aggregates (Lim et al., 2015). The activation of ULK1 specifically at aggregation sites would therefore create a positive feedback loop where p62 recruitment would promote ULK1 activation, which would in turn recruit and phosphorylate more p62. This would ensure rapid site-directed autophagosome formation around select cargo.

In addition to the regulation at the level of autophagosome formation, upstream starvation-dependent signals can also control the formation and autophagy-dependent degradation of protein aggregates. GSK3 is a kinase that regulates a wide variety of cellular processes ranging from metabolism to cell cycle and has been implicated in neurodegenerative diseases such as AD (Giese, 2009). GSK3 is phosphorylated and inhibited by AKT downstream of growth factor signaling. Interestingly, recent studies have shown that GSK3 inhibition promotes aggregation of ubiquitinated protein to presumably be cleared by selective autophagy (Carmichael et al., 2002; Nicot et al., 2014). In contrast, when GSK3 is activated, it phosphorylates the autophagic receptor NBR1, preventing aggregate formation and causing the accumulation of soluble ubiquitinated proteins (Nicot et al., 2014), suggesting that growth factor deprivation prevents the formation and subsequent autophagy-mediated degradation of aggregates. It should however be noted that inhibiting GSK3 improves cell survival in the context of a pathogenic mutant of Htt despite increasing aggregate accumulation, effect that was mapped to β-Cathenin/TCF-dependent transcription (Carmichael et al., 2002).

These discrepancies between aggregate accumulation and survival highlight the still unclear role of these large protein aggregates. While aggregate accumulation correlates with disease progression *in vivo*, several experiments suggest that they are in fact a protective mechanism required to sequester more toxic smaller oligomers (reviewed in Ben-Gedalya and Cohen, 2012). For example, neurons containing Htt aggregates or α-Synuclein inclusions survived better than the neighboring cells expressing the same toxic proteins without forming aggregates (Arrasate et al., 2004; Assayag et al., 2007). Therefore, since autophagy is clearly protective in the context of aggregate accumulation, its role in the clearance of the smaller oligomers is an important question that remains to be addressed. In addition, not all types of aggregate-prone proteins are readily degraded upon serum deprivation, some being resistant (Wong et al., 2008, 2012). In fact, even with the same aggregate-forming protein (synphilin-1), the sensitivity to serum withdrawal changes depending on whether a large aggresome or smaller aggregates are formed (Wong et al., 2012), possibly as a result of a failure to recruit the autophagy machinery to the resistant aggregates in spite of p62 recruitment (Wong et al., 2008). Altogether, these studies indicate that selective autophagy represents a large array of distinct but overlapping pathways to dispose of damaged or potentially toxic cellular content. Although a general picture of the mechanisms involved has emerged in recent years, the specific pathways regulating distinct types of cargo recognition and in fact the distinct nature of each cargo still remain for the most part elusive.

CONCLUSIONS

In the last decade, a wealth of data has indicated that activation of autophagy could be a valuable therapeutic avenue to treat neurodegenerative diseases. However, several important questions remain to more precisely target selective autophagy in this context. First, as starvation-induced autophagy and selective autophagy play distinct physiological roles that can lead to opposite outcomes in terms of survival, their contribution to neuronal survival needs to be properly established. Second, while the regulation of starvation-induced autophagy is relatively well understood (Mehrpour et al., 2010; Mizushima and Komatsu, 2011), the signaling pathways controlling selective autophagy still remain to be defined. Nevertheless, autophagy receptors such as p62 and NBR1 are likely to be central to this regulation as shown by the recent identification of several pathways regulating their function (Ichimura et al., 2013; Lim et al., 2015; Nicot et al., 2014; Ro et al., 2014). A third, more general question is the relationship between large aggregates, smaller oligomers, and autophagy. In particular, the role of autophagy in the degradation of smaller, potentially more toxic structures remains unknown.

As discussed in this review, while both forms of autophagy use the same core machinery to generate autophagosomes, some aspects of their regulation are distinct. This provides an opportunity to specifically manipulate one form or the other for therapeutic purposes. Therefore, given the therapeutic value of autophagy modulation in the treatment of diseases ranging from neurodegeneration to cancer, it will be crucial to define pathways uniquely associated with starvation-induced or selective autophagy to selectively target each.

Acknowledgments

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References

Abada, A., Elazar, Z., 2014. Getting ready for building: signaling and autophagosome biogenesis. EMBO Rep. 15, 839–852.

Alers, S., Loffler, A.S., Wesselborg, S., et al., 2012. The incredible ULKs. Cell Commun. Signal. 10, 7.

Arrasate, M., Mitra, S., Schweitzer, E.S., et al., 2004. Inclusion body formation reduces levels of mutant huntingtin and the risk of neuronal death. Nature 431, 805–810.

Assayag, K., Yakunin, E., Loeb, V., et al., 2007. Polyunsaturated fatty acids induce alpha-synuclein-related pathogenic changes in neuronal cells. Am. J. Pathol. 171, 2000–2011.

Ben-Gedalya, T., Cohen, E., 2012. Quality control compartments coming of age. Traffic 13, 635–642.

Berger, Z., Ravikumar, B., Menzies, F.M., et al., 2006. Rapamycin alleviates toxicity of different aggregate-prone proteins. Hum. Mol. Genet. 15, 433–442.

Bernard, A., Klionsky, D.J., 2013. Autophagosome formation: tracing the source. Dev. Cell 25, 116–117.

Birgisdottir, A.B., Lamark, T., Johansen, T., 2013. The LIR motif—crucial for selective autophagy. J. Cell Sci. 126, 3237–3247.

Bove, J., Martinez-Vicente, M., Vila, M., 2011. Fighting neurodegeneration with rapamycin: mechanistic insights. Nat. Rev. Neurosci. 12, 437–452.

Caccamo, A., Majumder, S., Richardson, A., et al., 2010. Molecular interplay between mammalian target of rapamycin (mTOR), amyloid-beta, and Tau: effects on cognitive impairments. J. Biol. Chem. 285, 13107–13120.

Caccamo, A., De Pinto, V., Messina, A., et al., 2014. Genetic reduction of mammalian target of rapamycin ameliorates Alzheimer's disease-like cognitive and pathological deficits by restoring hippocampal gene expression signature. J. Neurosci. 34, 7988–7998.

Carmichael, J., Sugars, K.L., Bao, Y.P., et al., 2002. Glycogen synthase kinase-3beta inhibitors prevent cellular polyglutamine toxicity caused by the Huntington's disease mutation. J. Biol. Chem. 277, 33791–33798.
Crews, L., Spencer, B., Desplats, P., et al., 2010. Selective molecular alterations in the autophagy pathway in patients with Lewy body disease and in models of alpha-synucleinopathy. PLoS One 5, e9313.
Duran, A., Amanchy, R., Linares, J.F., et al., 2011. p62 is a key regulator of nutrient sensing in the mTORC1 pathway. Mol. Cell 44, 134–146.
Filimonenko, M., Stuffers, S., Raiborg, C., et al., 2007. Functional multivesicular bodies are required for autophagic clearance of protein aggregates associated with neurodegenerative disease. J. Cell Biol. 179, 485–500.
Frake, R.A., Ricketts, T., Menzies, F.M., et al., 2015. Autophagy and neurodegeneration. J. Clin. Invest. 125, 65–74.
Giese, K.P., 2009. GSK-3: a key player in neurodegeneration and memory. IUBMB Life 61, 516–521.
Gomez-Suaga, P., Luzon-Toro, B., Churamani, D., et al., 2012. Leucine-rich repeat kinase 2 regulates autophagy through a calcium-dependent pathway involving NAADP. Hum. Mol. Genet. 21, 511–525.
Hara, T., Nakamura, K., Matsui, M., et al., 2006. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature 441, 885–889.
Hardie, D.G., 2014. AMP-activated protein kinase: a key regulator of energy balance with many roles in human disease. J. Intern. Med. 276, 543–559.
Heng, M.Y., Duong, D.K., Albin, R.L., et al., 2010. Early autophagic response in a novel knock-in model of Huntington disease. Hum. Mol. Genet. 19, 3702–3720.
Hochfeld, W.E., Lee, S., Rubinsztein, D.C., 2013. Therapeutic induction of autophagy to modulate neurodegenerative disease progression. Acta Pharmacol. Sin. 34, 600–604.
Ichimura, Y., Waguri, S., Sou, Y.S., et al., 2013. Phosphorylation of p62 activates the Keap1-Nrf2 pathway during selective autophagy. Mol. Cell 51, 618–631.
Isakson, P., Holland, P., Simonsen, A., 2013. The role of ALFY in selective autophagy. Cell Death Differ. 20, 12–20.
Itakura, E., Mizushima, N., 2010. Characterization of autophagosome formation site by a hierarchical analysis of mammalian Atg proteins. Autophagy 6, 764–776.
Jewell, J.L., Russell, R.C., Guan, K.L., 2013. Amino acid signalling upstream of mTOR. Nat. Rev. Mol. Cell. Biol. 14, 133–139.
Jimenez-Sanchez, M., Thomson, F., Zavodszky, E., et al., 2012. Autophagy and polyglutamine diseases. Prog. Neurobiol. 97, 67–82.
Kim, J., Kim, Y.C., Fang, C., et al., 2013. Differential regulation of distinct Vps34 complexes by AMPK in nutrient stress and autophagy. Cell 152, 290–303.
Koike, M., Shibata, M., Tadakoshi, M., et al., 2008. Inhibition of autophagy prevents hippocampal pyramidal neuron death after hypoxic-ischemic injury. Am. J. Pathol. 172, 454–469.
Komatsu, M., Waguri, S., Chiba, T., et al., 2006. Loss of autophagy in the central nervous system causes neurodegeneration in mice. Nature 441, 880–884.
Laplante, M., Sabatini, D.M., 2012. mTOR signaling in growth control and disease. Cell 149, 274–293.
Lee, J.Y., Koga, H., Kawaguchi, Y., et al., 2010. HDAC6 controls autophagosome maturation essential for ubiquitin-selective quality-control autophagy. EMBO J. 29, 969–980.
Levine, B., Packer, M., Codogno, P., 2015. Development of autophagy inducers in clinical medicine. J. Clin. Invest. 125, 14–24.
Li, Y., Chopp, M., Jiang, N., et al., 1995. Temporal profile of in situ DNA fragmentation after transient middle cerebral artery occlusion in the rat. J. Cereb. Blood Flow Metab. 15, 389–397.
Lim, J., Lachenmayer, M.L., Wu, S., et al., 2015. Proteotoxic stress induces phosphorylation of p62/SQSTM1 by ULK1 to regulate selective autophagic clearance of protein aggregates. PLoS Genet. 11, e1004987.
Lin, X., Li, S., Zhao, Y., et al., 2013. Interaction domains of p62: a bridge between p62 and selective autophagy. DNA Cell Biol. 32, 220–227.
Lipinski, M.M., Hoffman, G., Ng, A., et al., 2010. A genome-wide siRNA screen reveals multiple mTORC1 independent signaling pathways regulating autophagy under normal nutritional conditions. Dev. Cell 18, 1041–1052.
Lomonosova, E., Chinnadurai, G., 2008. BH3-only proteins in apoptosis and beyond: an overview. Oncogene 27 (Suppl. 1), S2–19.
Lynch-Day, M.A., Mao, K., Wang, K., et al., 2012. The role of autophagy in Parkinson's disease. Cold Spring Harb. Perspect. Med. 2, a009357.
Maiuri, M.C., Le Toumelin, G., Criollo, A., et al., 2007. Functional and physical interaction between Bcl-X(L) and a BH3-like domain in Beclin-1. EMBO J. 26, 2527–2539.

Majumder, S., Richardson, A., Strong, R., et al., 2011. Inducing autophagy by rapamycin before, but not after, the formation of plaques and tangles ameliorates cognitive deficits. PLoS One 6, e25416.
Martinez-Vicente, M., Talloczy, Z., Wong, E., et al., 2010. Cargo recognition failure is responsible for inefficient autophagy in Huntington's disease. Nat. Neurosci. 13, 567–576.
McEwan, D.G., Dikic, I., 2011. The three musketeers of autophagy: phosphorylation, ubiquitylation and acetylation. Trends Cell Biol. 21, 195–201.
Mehan, S., Meena, H., Sharma, D., et al., 2011. JNK: a stress-activated protein kinase therapeutic strategies and involvement in Alzheimer's and various neurodegenerative abnormalities. J. Mol. Neurosci. 43, 376–390.
Mehrpour, M., Esclatine, A., Beau, I., et al., 2010. Overview of macroautophagy regulation in mammalian cells. Cell Res. 20, 748–762.
Menzies, F.M., Huebener, J., Renna, M., et al., 2010. Autophagy induction reduces mutant ataxin-3 levels and toxicity in a mouse model of spinocerebellar ataxia type 3. Brain 133, 93–104.
Menzies, F.M., Garcia-Arencibia, M., Imarisio, S., et al., 2015. Calpain inhibition mediates autophagy-dependent protection against polyglutamine toxicity. Cell Death Differ. 22, 433–444.
Mihaylova, M.M., Shaw, R.J., 2011. The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. Nat. Cell Biol. 13, 1016–1023.
Mizushima, N., Komatsu, M., 2011. Autophagy: renovation of cells and tissues. Cell 147, 728–741.
Nair, U., Yen, W.L., Mari, M., et al., 2012. A role for Atg8-PE deconjugation in autophagosome biogenesis. Autophagy 8, 780–793.
Nakatogawa, H., Ishii, J., Asai, E., et al., 2012. Atg4 recycles inappropriately lipidated Atg8 to promote autophagosome biogenesis. Autophagy 8, 177–186.
Narendra, D., Kane, L.A., Hauser, D.N., et al., 2010a. p62/SQSTM1 is required for Parkin-induced mitochondrial clustering but not mitophagy; VDAC1 is dispensable for both. Autophagy 6, 1090–1106.
Narendra, D.P., Jin, S.M., Tanaka, A., et al., 2010b. PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. PLoS Biol. 8, e1000298.
Nicot, A.S., Lo Verso, F., Ratti, F., et al., 2014. Phosphorylation of NBR1 by GSK3 modulates protein aggregation. Autophagy 10, 1036–1053.
Ozcelik, S., Fraser, G., Castets, P., et al., 2013. Rapamycin attenuates the progression of tau pathology in P301S tau transgenic mice. PLoS One 8, e62459.
Pandey, U.B., Nie, Z., Batlevi, Y., et al., 2007. HDAC6 rescues neurodegeneration and provides an essential link between autophagy and the UPS. Nature 447, 859–863.
Pickford, F., Masliah, E., Britschgi, M., et al., 2008. The autophagy-related protein Beclin 1 shows reduced expression in early Alzheimer disease and regulates amyloid beta accumulation in mice. J. Clin. Invest. 118, 2190–2199.
Proenca, C.C., Stoehr, N., Bernhard, M., et al., 2013. Atg4b-dependent autophagic flux alleviates huntington's disease progression. PLoS One 8, e68357.
Ravikumar, B., Duden, R., Rubinsztein, D.C., 2002. Aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy. Hum. Mol. Genet. 11, 1107–1117.
Ravikumar, B., Vacher, C., Berger, Z., et al., 2004. Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. Nat. Genet. 36, 585–595.
Ro, S.H., Semple, I.A., Park, H., et al., 2014. Sestrin2 promotes Unc-51-like kinase 1 mediated phosphorylation of p62/sequestosome-1. FEBS J. 281, 3816–3827.
Rogov, V., Dotsch, V., Johansen, T., et al., 2014. Interactions between autophagy receptors and ubiquitin-like proteins form the molecular basis for selective autophagy. Mol. Cell 53, 167–178.
Rose, C., Menzies, F.M., Renna, M., et al., 2010. Rilmenidine attenuates toxicity of polyglutamine expansions in a mouse model of Huntington's disease. Hum. Mol. Genet. 19, 2144–2153.
Shibata, M., Lu, T., Furuya, T., et al., 2006. Regulation of intracellular accumulation of mutant Huntingtin by Beclin 1. J. Biol. Chem. 281, 14474–14485.
Siesjo, B.K., Katsura, K., Zhao, Q., et al., 1995. Mechanisms of secondary brain damage in global and focal ischemia: a speculative synthesis. J. Neurotrauma 12, 943–956.
Spencer, B., Potkar, R., Trejo, M., et al., 2009. Beclin 1 gene transfer activates autophagy and ameliorates the neurodegenerative pathology in alpha-synuclein models of Parkinson's and Lewy body diseases. J. Neurosci. 29, 13578–13588.
Spilman, P., Podlutskaya, N., Hart, M.J., et al., 2010. Inhibition of mTOR by rapamycin abolishes cognitive deficits and reduces amyloid-beta levels in a mouse model of Alzheimer's disease. PLoS One 5, e9979.

Suzuki, H., Tabata, K., Morita, E., et al., 2014. Structural basis of the autophagy-related LC3/Atg13 LIR complex: recognition and interaction mechanism. Structure 22, 47–58.
Vakifahmetoglu-Norberg, H., Xia, H.G., Yuan, J., 2015. Pharmacologic agents targeting autophagy. J. Clin. Invest. 125, 5–13.
Wang, T., Lao, U., Edgar, B.A., 2009. TOR-mediated autophagy regulates cell death in *Drosophila* neurodegenerative disease. J. Cell Biol. 186, 703–711.
Webb, J.L., Ravikumar, B., Atkins, J., et al., 2003. Alpha-synuclein is degraded by both autophagy and the proteasome. J. Biol. Chem. 278, 25009–25013.
Wei, Y., Pattingre, S., Sinha, S., et al., 2008. JNK1-mediated phosphorylation of Bcl-2 regulates starvation-induced autophagy. Mol. Cell 30, 678–688.
Wen, Y.D., Sheng, R., Zhang, L.S., et al., 2008. Neuronal injury in rat model of permanent focal cerebral ischemia is associated with activation of autophagic and lysosomal pathways. Autophagy 4, 762–769.
White, E., 2015. The role for autophagy in cancer. J. Clin. Invest. 125, 42–46.
Williams, A., Sarkar, S., Cuddon, P., et al., 2008. Novel targets for Huntington's disease in an mTOR-independent autophagy pathway. Nat. Chem. Biol. 4, 295–305.
Winslow, A.R., Chen, C.W., Corrochano, S., et al., 2010. Alpha-synuclein impairs macroautophagy: implications for Parkinson's disease. J. Cell Biol. 190, 1023–1037.
Wong, E., Bejarano, E., Rakshit, M., et al., 2012. Molecular determinants of selective clearance of protein inclusions by autophagy. Nat. Commun. 3, 1240.
Wong, E.S., Tan, J.M., Soong, W.E., et al., 2008. Autophagy-mediated clearance of aggresomes is not a universal phenomenon. Hum. Mol. Genet. 17, 2570–2582.
Xia, H.G., Zhang, L., Chen, G., et al., 2010. Control of basal autophagy by calpain1 mediated cleavage of ATG5. Autophagy 6, 61–66.
Xing, S., Zhang, Y., Li, J., et al., 2012. Beclin 1 knockdown inhibits autophagic activation and prevents the secondary neurodegenerative damage in the ipsilateral thalamus following focal cerebral infarction. Autophagy 8, 63–76.
Yamamoto, A., Simonsen, A., 2011. Alfy-dependent elimination of aggregated proteins by macroautophagy: can there be too much of a good thing? Autophagy 7, 346–350.
Yu, Z.Q., Ni, T., Hong, B., et al., 2012. Dual roles of Atg8-PE deconjugation by Atg4 in autophagy. Autophagy 8, 883–892.
Zhang, L., Yu, J., Pan, H., et al., 2007. Small molecule regulators of autophagy identified by an image-based high-throughput screen. Proc. Natl. Acad. Sci. U.S.A. 104, 19023–19028.
Zhang, X., Yan, H., Yuan, Y., et al., 2013. Cerebral ischemia-reperfusion-induced autophagy protects against neuronal injury by mitochondrial clearance. Autophagy 9, 1321–1333.

CHAPTER

6

Molecular Mechanisms Underlying Cell Death Caused by Cationic Polymers

Jung-Hua Steven Kuo, Ming-Shiou Jan and Chia-Wei Lin

OUTLINE

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Abstract

Cationic polymers have frequently been used as nonviral vectors for delivering nucleic acids. Despite their fairly high transfection activity, the well-known cytotoxicity of cationic polymers remains a key obstacle for their use in clinical applications. Due to the dynamic and complicated characteristics of cell death, interpretations of the cell death mechanisms induced by cationic polymers have been inconclusive and the exact molecular mechanisms of cell death induced by cationic polymers are not generally well understood. Therefore, the aim of this review is to discuss the molecular mechanisms underlying cell death caused by cationic polymers, with hope that it may provide a deeper insight into those mechanisms.

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INTRODUCTION

The utility of cationic polymers in nucleic acid delivery makes them an attractive means for some therapeutic applications. Cationic polymers, which are classified as nonviral vectors, are capable of complexing with and condensing negatively charged nucleic acids. The resultant cationic polymer–nucleic acid complexes are internalized into cells mainly by endocytosis, allowing them to overcome the entrapment and degradation of nucleic acids in endosomes, and eventually helping DNA to enter the cell nucleus or shuffle RNA to target sites (Gupta et al., 2012). In comparison with viral vectors, cationic polymers have some favorable characteristics as gene delivery systems, such as a lack of any specific immune response, the ease with which they can be used for scale-up production, well-defined structural properties, and greater flexibility for delivering larger pieces of genetic material. However, the well-known cytotoxicity of cationic polymers remains a key obstacle to their clinical use (Lv et al., 2006). Furthermore, due to the dynamic and complicated characteristics of cell death, interpretations of cell death mechanisms induced by cationic polymers have been inconclusive. Therefore, the aim of this review is to investigate the molecular mechanisms underlying cell death caused by cationic polymers, in the hopes that understanding the consequences induced by cationic polymers will provide helpful information for safer designs in delivering therapeutic nucleic acids.

CHARACTERISTICS OF CATIONIC POLYMERS

There are a variety of cationic polymers that have been developed for nucleic acid delivery (Morille et al., 2008). Here, we mainly focus on cationic polymers that are commonly used, which include poly(ethylenimine) (PEI), poly(L-lysine) (PLL), and dendrimers. PEI polymers are the most commonly used vectors in cationic polymer gene delivery systems. Compared with other cationic polymers, PEI exhibits better transfection efficiency in various cell lines (Morille et al., 2008). Due to the differing pK_a values of non-protonated amines, PEI polymers provide significant buffering capacity over other cationic polymers at the low pH levels of the lysosomes. This large buffering capacity of PEI results in the escape of polymer/nucleic acid complexes from endosomes, a process which is known as the proton-sponge effect. Various forms of PEI with linear or branched structures and with different molecular weights have been made commercially available. However, high amounts of positive amines cause high toxicity of PEI *in vitro* or *in vivo* (Godbey et al., 1999; Wang et al., 2011).

PLL polymers are biodegradable due to the peptide construct of PLL and thus are useful for *in vivo* gene delivery. The transfection efficiency of PLL polymers is associated with the molecular weight of PLL (Mannisto et al., 2002). PLL polymers with high molecular weights form stable complexes with DNA and the transfection efficiency and cytotoxicity are increased due to the high molecular weight of PLL polymers. Although PLL polymers can condense DNA into small particles and facilitate cellular uptake via endocytosis, PLL polymers lack the buffering capacity and the proton-sponge effect necessary for lysosomal escape.

Dendrimers are a family of highly branched polymers with well-defined structures. Dendrimers have narrow polydispersity, high surface functional groups, and open inner spaces for drug entrapment (Shcharbin et al., 2010). Cationic dendrimers such as

poly(propylene imine) (PPI) and poly(amidoamine) (PAMAM) have been applied for nucleic acid delivery and diverse biomedical applications (Shcharbin et al., 2010). The transfection efficiencies of gene delivery when using dendrimers depend greatly on their molecular structures. Activated dendrimers are achieved by solvolytic heat-degradation treatment of dendrimers and exhibit more flexible structures and higher transfection efficiency (Navarro and de ILarduya, 2009). Also, cationic PPI dendrimers have shown significant antitumor effects *in vivo* (Dufès et al., 2005). Usually, dendrimers with higher generation exhibit more cytotoxicities than those with lower generation (Shcharbin et al., 2010).

BRIEF OVERVIEW OF MOLECULAR MECHANISMS OF CELL DEATH

Morphologically, cell death is related to at least four distinct processes: apoptosis (type 1 cell death), autophagy (type 2 cell death), necrosis (type 3 cell death), and pyroptosis (Mizushima et al., 2010; Bergsbaken et al., 2009). Necrosis and pyroptosis are related to inflammatory and degenerative processes. Cells undergoing necrosis characteristically exhibit mitochondrial swelling, decreased membrane integrity, inhibited metabolism, and the release of cytoplasmic components that stimulate an inflammatory response (Mizushima et al., 2010). Except for harsh conditions encountered in cells, programmed necrotic cell death has been explored and some related molecules such as R1P1 and CypD have been identified as being involved in necrotic signaling pathways. Pyroptosis is a form of caspase-1 dependent programmed cell death that results in the rapid lysis of the cells and the release of inflammatory cytokines such as IL-1β and IL-18 (Bergsbaken et al., 2009). In contrast to necrosis and pyroptosis, apoptosis, or shrinkage necrosis, is a form of programmed cell death that is characterized by cytoplasmic blebbing, condensation of the nuclear chromatin, cell shrinkage, DNA fragmentation, exposure of phosphatidylserine residues on the outer leaflet, and cellular fragmentation into membrane apoptotic bodies (Mukhopadhyay et al., 2014). Generally, the apoptotic signaling pathways are classified as extrinsic and intrinsic pathways (Mukhopadhyay et al., 2014). The extrinsic apoptotic pathway is triggered by the interaction between specific ligands and plasma membrane death receptors such as the tumor necrosis factor receptor (TNFR) superfamily. The stimulation of death receptors then activates the initiator caspases (caspase-8 or caspase-10 in humans) to form a death-inducing signaling complex which further transmits death signals. The proteolysis of the B-cell lymphoma 2 (BCL2) homology-3 (BH3)-only protein BID then increases mitochondrial outer membrane permeabilization (MOMP), the direct proteolytic activation of downstream effector caspases, or the activation of the kinase RIP for the further execution of apoptosis induction. In the intrinsic apoptosis pathway, intracellular stimuli initiate MOMP which in turn causes the release of mitochondrial cytochrome c (Cyt c). In the cytosol, Cyt c triggers the formation of apoptosome for further caspase activation.

Autophagy is a conserved cell pathway and it has been associated with several physiological and pathological processes. Autophagy starts with the formation of phagophores, which are double-layered and crescent-shaped membranes for the stepwise engulfment of cytoplasmic material. The phagophores then mature into autophagosomes and finally are delivered to lysosomes for degradation. The activation of autophagy engages a series of cellular processes: regulation of induction, vesicle nucleation, vesicle elongation, retrieval,

docking and fusion, and vesicle breakdown and degradation (Mukhopadhyay et al., 2014). Autophagy involves a set of autophagy-related proteins (*Atg*) that are required to form the isolation membrane and the autophagosome (Mizushima et al., 2010). LC3, a mammalian homologue of Atg8, was originally identified as a microtubule-associated protein 1 light chain 3 and is widely used as a marker for autophagosomes. After synthesis, an unprocessed form of LC3 is cleaved by Atg4 protease and becomes LC3-I. LC3-II is formed after LC3-I conjugates with phosphatidylethanolamine and remains associated with the autophagosomes. The conversion of cytosolic LC3-I to LC3-II located on the autophagosome membrane is a useful and sensitive marker for detecting autophagy in mammalian cells (Mizushima et al., 2010). The increase in LC3-II can be associated with either an increase in the formation of autophagosomes or impaired autophagic degradation (Mizushima et al., 2010). Furthermore, polyubiquitin-binding p62 protein (sequestosome 1 (SQSTM1)) binds to LC3 and is selectively recognized and degraded by autophagy (Mizushima, 2007). Ubiquitinated protein aggregates and inclusion bodies can be recruited to the autophagosome membrane by p62. Beclin 1, which is the mammalian orthologue of yeast Atg6, plays an important role in autophagy (Mizushima et al., 2010). The induction of autophagy requires various interactions among beclin 1, mammalian Vps34 (a class III phosphatidylinositol 3-kinase (PI3K)), UVRAG (UV irradiation resistance-associated tumor suppressor gene), and Bcl2/Bcl-XL. Beclin 1 interacts with apoptosis proteins such as Bcl2 or Bcl-XL, resulting in crosstalk between apoptosis and autophagy.

MOLECULAR MECHANISMS OF CELL DEATH CAUSED BY CATIONIC POLYMERS

Interpretations of cell death mechanisms induced by cationic polymers have been inconsistent and not entirely clear due to the dynamic and complicated process of cell death. The cytotoxic mechanisms of cationic polymers may be triggered by the interactions of cationic polymers with cell membranes or by the activation of intracellular signal transduction pathways from cellular uptake. PLL induces cellular efflux of organic and inorganic molecules in tumor cells after membrane adsorption (Arnold et al., 1979). However, in human vascular endothelial cells, the cytotoxicity of PLL has been found to not be affected by the inhibition of cellular microtubules and microfilament formation (Morgan et al., 1989). Usually, the complexes of cationic polymers and DNA are less cytotoxic than uncomplexed cationic polymers due to decreases in the positive charges of the cationic polymers. Using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and LDH (lactate dehydrogenase) assays, Fischer et al. (2003) demonstrated that the cytotoxicity of PEI was comparable with PLL and that the starburst dendrimer is less cytotoxic than PEI and PLL in L929 mouse fibroblasts. They further found that PEI and its DNA complexes induce the necrotic type of cell death in SW13 carcinoma cells based on the observations of early membrane leakage and the failure of the inhibition by the caspase inhibitor zVAD.fmk (Fischer et al., 2003). Branched and linear PEI polymers and the DNA complexes of PEI have been shown to firstly induce membrane damage and then to initiate apoptosis in Jurkat T cells, umbilical vein endothelial cells, and THLE3 hepatocyte-like cells (Hunter, 2006). With a brief amount of incubation time 30 min, PEI polymers induce characteristics of necrosis such as

substantial LDH release and phosphatidylserine translocation from the inner plasma membrane to the outer cell surface. With a longer period of incubation (24h), PEI induces channel formation in the outer mitochondrial membrane and initiates mitochondrially mediated apoptosis. The release of proapoptotic Cyt c, the activation of caspase 3, and alterations in mitochondrial membrane potential have been demonstrated in PEI-treated Jurkat T cells, and these apoptotic activities have further been found to be inhibited by the presence of the caspase-3 inhibitor Ac-DEVD-CHO (Hunter, 2006). Recently, PEI and PEI/RNA (DNA) complexes have been shown to induce autophagic cell death in various cells (Alonso-Curbelo and Soengas, 2010; Gao et al., 2011; Lin et al., 2012; Roberts et al., 2013). PEI conjugated with dsRNA mimics polyinosine-polycytidylic acid (pIC) induced self-degradation by autophagy and apoptosis via endo/lysosomal machinery in melanoma cells (Alonso-Curbelo and Soengas, 2010). The RNA helicase MDA-5 (melanoma differentiation-associated gene-5) has been found to be activated by PEI-pIC complexes, leading to the promotion of the proapoptotic factor NOXA with subsequent processing of apoptotic caspases for anti-melanoma activity. PEI has also been shown to induce autophagy in HeLa cancer cells, and the occurrence of apoptosis and autophagy may be independent (Lin et al., 2012). Furthermore, autophagy has been determined to play a role in the cell survival of PEI-treated cells based on the fact that PEI induces a higher degree of cell death in $atg5^{-/-}$ mouse embryonic fibroblast cells than in wild-type cells. In contrast, 3-MA has been reported to inhibit autophagy and to reduce cell cytotoxicity in PEI-treated MDCK and Chang liver cells, indicating that autophagy promotes cell death (Gao et al., 2011). PEI/DNA complexes have been demonstrated to enter various cells in endosomes and to activate the formation of tubulovesicular autophagosomes from small autophagosomes (Roberts et al., 2013). Gene expression has been shown to be higher in $atg5^{-/-}$ cells treated with PEI/DNA complexes, indicating that tubulovesicular autophagosomes can be considered as a new barrier for gene delivery.

In three human cell lines, low- and high-molecular-weight PLL polymers and their complexes with DNA have also been found to induce two stages of cytotoxicity, namely, early cell membrane damage followed by apoptosis for a longer period of incubation (24h) (Hunter, 2006). Low- and high-molecular-weight PLL polymers trigger mitochondrial-mediated apoptotic pathways, as evidenced by Cyt c release, caspase 3 and caspase 9 activation, and mitochondrial membrane potential changes in Jurkat T-cells. High-molecular-weight PLL polymers induce more caspase 3 activation and Cyt c release than low-molecular-weight PLL polymers with longer periods of incubation (24h). However, genotoxic stress signal caspase 2 is not activated after treatment of PLL polymers. Also, high-molecular-weight PLL polymers have been found to reduce Cyt c release in the presence of the Bax channel blocker but low-molecular-weight PLL polymers have been found to not be affected by the Bax channel blocker. This indicates that the apoptotic pathways of the two PLL polymers are different. Moreover, high but not low-molecular-weight PLL polymers have been found to induce membrane depolarization and a progressive drop in the rate of uncoupled respiration in isolated rat liver mitochondria. Notably, branched PEI has been determined to have no direct effects on the above mitochondrial physiological functions. After cellular uptake, free PEI exists in the cytoplasm and later becomes accumulated within lysosomes (Godbey et al., 1999). PEI induces cathepsin release by disrupting the lysosomal membrane from the cytoplasmic side (Klemm et al., 1998). The cytoplasmic cathepsins can cleave the Bcl2 family member Bid and the antiapototic Bcl2 homologues, resulting in the formation of pores in the

outer mitochondrial membrane that assist the release of Cyt c (Droga-Mazovec et al., 2008). The apoptotic process is triggered by the oligomeric apoptosome that is formed after Cyt c interacts with the apoptotic protease activating factor 1 (Apaf1) and then cleaves pro-caspase 9 to active caspase 9, which then further activates caspase 3 (Hunter and Moghimi, 2010). PLL has also been shown, based on the evidence of fluorescent puncta formations, conversions of LC3-I to LC3-II, and p62 degradation, to induce autophagy in HeLa cells (Lin et al., 2012). The degree of cell death has been found to be higher in incubated HeLa cells treated with PLL plus autophagy inhibitors, indicating the protective role of autophagy in PLL-treated cells.

Similarly, PAMAM dendrimers have been shown to induce necrosis and apoptosis in RAW 264.7 murine macrophage-like cells (Kuo et al., 2005). PAMAM dendrimers have likewise been shown to induce mitochondrial-mediated apoptosis by activating caspase 3 and caspase 9 followed by the release of Cyt c (Lee et al., 2009). However, cationic PAMAM dendrimers have also been reported to promote acute lung injury in mice by inducing autophagic cell death through the Akt-TSC2-mTOR signaling pathway (Li et al., 2009). Cationic PAMAM also induces LC3 aggregation and increases of LC3-II expression in A549 cells. The nonspecific autophagic inhibitor 3-methyladenine reduces acute lung injury *in vivo* and cell death in the human lung adenocarcinoma A549 cells induced by cationic dendrimers. PPI dendrimers, meanwhile, have been shown to induce different kinetic responses of the cellular reactive oxygen species (ROS) and that PPI dendimers are also dependent on the generation of the dendrimer (Kuo et al., 2007). ROS are important in apoptosis because they modulate multiple signaling pathways and transcriptional activation. Similarly, mitochondrial membrane potential in macrophages treated with dendrimers has been demonstrated to also be dependent on dendrimer generation. Mitochondria are both the source and target of ROS and are also responsible for producing cellular energy. Apoptotic activation of mitochondria leads to a disturbance of mitochondrial membrane potential, permeability transition, the generation of ROS, and the release of apoptotic proteins. Also, macrophages treated with PPI dendrimers exhibit dramatic changes in cell size and complexity. Increases in the subG1 population of cells treated with dendrimers have also been demonstrated, and this finding indicates that the changes of ROS generation and mitochondrial membrane potential strongly influence cell proliferation and cause apoptosis in cells incubated with dendrimers.

The gene expression profiles of cell death induced by cationic polymers, which can provide upstream information regarding related proteins, have also been investigated. Gene profiling on toxicity pathways of PEI-based polymers for pulmonary applications has been conducted, and the results demonstrate that 25 KDa PEI induces the intrinsic apoptotic pathway in murine alveolar epithelial–like type II LA4 cells after 24 h of treatment (Beyerle et al., 2010). Besides the apoptotic genes (*Bcl2l1*, *Bax*, *Anxa5*, *Ercc4*, *and Atm*), which were induced after 6 h of incubation, a cell cycle regulating transcription factor (*E2f1*), the gene involved in proinflammatory and apoptosis/survival signaling pathways (*NfkBia*), and the gene related with oxidative stress indicating phase II metabolizing enzyme (*Ugt1a2*) were also induced for 25 KDa PEI. However, no significant release from LA4 cells of proinflammatory cytokines (IL-1β, IL-6, TNF-alpha, G-CSF, CXCL1 CXCL10, CCL2, and CCL3) was detected for 25 KDa PEI with 24 h treatment. In another study, the genes related to anti-apoptotic responses—that is, the Bcl2 family, Caspase family, CARD family, and TRAF

family—have been strongly induced in H1299/Luc (NCI-H1299 immortalized nonsmall cell lung cancer cells with luciferase reporter gene) cells treated with 25 KDa PEI after 24 h of treatment (Merkel et al., 2011). The genes related to the caspase recruitment domain (CARD) family (BIRC2, BIRC3, CARD6, and CARD8) were up-regulated after 24 h of treatment and the genes involved in the inhibitor of apoptosis (IAP) family (BIRC2, BIRC3, BIRC4 (XIAP), BIRC6, and BIRC8) were also up-regulated after 24 h treatment, even though these same genes were down-regulated after only 6 h of incubation. The genes of the Bcl2 family and the genes related to the DNA damage response were either up- or down-regulated after 24 h of treatment, while the genes of caspase 2 and caspase 8 were down-regulated after 6 and 24 h of treatment. The genes of other caspases were up-regulated after 24 h of treatment, indicating that mitochondrial death rather than death receptor-mediated apoptosis was involved. However, the gene profile of PEI seems to be cell line specific. Furthermore, it has been demonstrated that PEI resulted in up-regulation of CMV-promoter driven reporter genes in stably transfected cells through the activation of NF-kB.

Besides *in vitro* cell culture systems, linear and branched PEI have also been found to induce various gene changes in A431 nude mice with tumor xenografts by intratumoral administration, and branched PEI was also found to induce more gene changes than the linear form of PEI (Akhtar and Benter, 2007). Intratracheal administration of complexes of PEI/DNA in Balb/c mice has been found to induce changes in the genes involved in inflammatory processes, DNA damage, the cell cycle, apoptosis, and DNA repair (Regnström et al., 2006). The gene expression profiles of PEI alone and complexes with plasmid DNA from spleen lymphocytes after *in vivo* immunization of mice have also been studied (Regnström et al., 2003). The genes induced by PEI alone were involved in various cell toxic processes such as cell cycle regulation, oncogenesis, and differentiation. Complexes of PEI/DNA have likewise been found to activate apoptotic FasL, which is related to cytotoxic T-cell-mediated apoptosis and is involved in the antigen-stimulated suicide of mature T cells. The up-regulation of FasL by these complexes has been shown to induce the expression of TRAIL, which suggests that the FasL-mediated death pathway is involved.

Since cells may respond in a flexible manner to cell death stimuli and this may result in different expression profiles, assays at the single cell level such as flow cytometry have been applied in PEI-treated cells (Martin et al., 2013). A coordinated induction of genes related to cell stress and the cell cycle have been demonstrated in transfected HEK 293 T cells treated with complexes of PEI and pEGFPLuc plasmid encoding for green fluorescent protein (GFP).

The toxicogenomics of cationic dendrimers have previously been reported (Omidi et al., 2005; Akhtar and Benter, 2007; Kuo et al., 2010). The effects of cationic PPI dendrimers on gene expression profiles have been demonstrated to depend on dendrimer generations, where the number of genes affected is greater with higher generation. The gene expression profiles have also been shown to be different between cationic dendrimers alone and complexes of cationic dendrimers/DNA. Furthermore, the genes induced by cationic dendrimers in A431 epithelial cells have been shown to be involved in defense responses, cell proliferation, and apoptosis, with a diverse range of genes being affected, including anxa2, Bcl2a1, tnfrsf7, cdk4, dusp 1, cdc2, h2afg, hspd1, psma4, alox5, gmps, and il5 (Omidi et al., 2005). Although similar genes related to apoptosis, cell proliferation, and cytokine signaling

pathways were affected, the patterns of gene expression of complexes of cationic dendrimers/DNA were different than those of cationic dendrimers alone. In A549 lung cells treated with PPI generation 3 (DAB-16) alone and complexes of DAB-16/DNA, the expression ratios of tgfb1, Bcl2a1, cxcr4, prkca, chek1, csk, cdo1, dusp1, and srpr were altered by complexes of DAB-16/DNA but not by DAB-16 alone. In addition to inducing gene expression profiles related to cell death mechanisms by cationic polymers, the "off-target" effects of the delivery of small interfering RNA (siRNA) by cationic polymers have been demonstrated (Akhtar and Benter, 2007; Merkel et al., 2011). The siRNA activity in cells may be compromised or enhanced if cationic polymers also alter the expression of genes targeted for silencing.

Furthermore, the differences in structures of cationic dendrimers have been found to induce the different patterns of gene changes and also to alter target gene expression for delivery siRNA (Hollins et al., 2007). Two PAMAM dendrimers (Superfect and Polyfect) have been shown to induce different gene expression changes, including opposing effects on the expression of epidermal growth factor receptor (EGFR) in the human epidermoid carcinoma A431 cell line (Hollins et al., 2007). The nature of the nucleic acid (DNA or siRNA) alone, which is not in complexes form with PAMAM dendrimers, has been shown to determine the degree of total gene changes and siRNA/dendrimer complexes had a greater tendency for off-target effects than DNA/dendrimer complexes. The expression of EGFR mRNA in cells treated with Superfect dendrimers was increased approximately twofold, whereas Polyfect and siRNA/dendrimer complexes inhibited EGFR mRNA by approximately half. The cellular uptakes for these two types of dendrimer/siRNA complexes were comparable, but Polyfect/anti-EGFR siRNA complexes inhibited more potent gene silencing at the EGFR protein level. In contrast, branched form of PEI has been shown to enhance EGFR expression in A431 cells, while the silencing effects induced by PEI/anti-EGFR siRNA complexes were less pronounced than those induced by cationic lipid systems (Akhtar and Benter, 2007). The changes in global gene-expression profiles in human cervical cancer HeLa cells exposed to nonactivated and activated PAMAM dendrimers, alone or in complexes with plasmid DNA, have previously been demonstrated (Kuo et al., 2010). Activated and nonactivated dendrimers and their complexes with DNA alike induced multiple gene expression changes, some of which overlapped with their complexes. Dendrimer activation improved transfection efficiency and induced additional gene expression changes in HeLa cells. Three genes related to survival signaling and the proliferation of cancer cells (PHF5A, ARNTL2, and CHD4) have been identified. Finally, pyroptosis has not been reported in cells treated with cationic dendrimers, PEI, and PLL.

CONCLUSIONS

Cationic polymers induce multiple cytotoxic effects *in vivo* and *in vitro*. Determining the mechanisms of cell death caused by cationic polymers is very important, and *in vivo* tests are critical way to gain some insights. The study of the molecular mechanisms of cell death induced by cationic polymers is complicated due to the dynamic and intricate characteristics of cell death. However, understanding the molecular mechanisms of cell death induced by cationic polymers will be helpful in the design of more safe and efficient nonviral vectors for nucleic acid delivery.

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References

Akhtar, S., Benter, I., 2007. Toxicogenomics of non-viral drug delivery systems for RNAi: potential impact on siRNA-mediated gene silencing activity and specificity. Adv. Drug Deliv. Rev. 59, 164–182.

Alonso-Curbelo, D., Soengas, M.S., 2010. Self-killing of melanoma cells by cytosolic delivery of dsRNA: wiring innate immunity for a coordinated mobilization of endosomes, autophagosomes and the apoptotic machinery in tumor cells. Autophagy 6, 148–150.

Arnold, L.J., Dagan, A., Gutheil, J., et al., 1979. Antineoplastic activity of poly (L-lysine) with some ascites tumor cells. Proc. Natl. Acad. Sci. 76, 3246–3250.

Bergsbaken, T., Fink, S.L., Cookson, B.T., 2009. Pyroptosis: host cell death and inflammation. Nat. Rev. Microbiol. 7, 99–109.

Beyerle, A., Irmler, M., Beckers, J., et al., 2010. Toxicity pathway focused gene expression profiling of PEI-based polymers for pulmonary applications. Mol. Pharm. 7, 727–737.

Droga-Mazovec, G., Bojič, L., Petelin, A., et al., 2008. Cysteine cathepsins trigger caspasesdependent cell death through cleavage of Bid and antiapototic Bcl-2 homologues. J. Biol. Chem. 283, 19140–19150.

Dufès, C., Uchegbu, I.F., Schätzlein, A.G., 2005. Dendrimers in gene delivery. Adv. Drug Deliv. Rev. 57, 2177–2202.

Fischer, D., Li, Y., Ahlemeyer, B., et al., 2003. *In vitro* cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. Biomaterials 24, 1121–1131.

Gao, X., Yao, L., Song, Q., et al., 2011. The association of autophagy with polyethylenimine-induced cytotoxicity in nephritic and hepatic cell lines. Biomaterials 32, 8613–8625.

Godbey, W.T., Wu, K.K., Mikos, A.G., 1999. Size matters: molecular weight affects the efficiency of poly(ethylenimine) as a gene delivery vehicle. J. Biomed. Mater. Res. A. 45, 268–275.

Gupta, M., Tiwari, S., Vyas, S., 2012. Structuring polymers for delivery of DNA-based therapeutics: updated insights. Crit. Rev. Ther. Drug Carrier Syst. 29, 447–485.

Hollins, A.J., Omidi, Y., Benter, I.F., et al., 2007. Toxicogenomics of drug delivery systems: exploiting delivery system-induced changes in target gene expression to enhance siRNA activity. J. Drug Target. 15, 83–88.

Hunter, A.C., 2006. Molecular hurdles in polyfectin design and mechanistic background to polycation induced cytotoxicity. Adv. Drug Deliv. Rev. 58, 1523–1531.

Klemm, A.R., Young, D., Lloyd, J.B., 1998. Effects of polyethylenimine on endocytosis and lysosome stability. Biochem. Pharmacol. 56, 41–46.

Kuo, J.H., Jan, M.S., Chiu, H.W., 2005. Mechanism of cell death induced by cationic dendrimers in RAW 264.7 murine macrophage-like cells. J. Pharm. Pharmacol. 57, 489–495.

Kuo, J.H., Jan, M.S., Lin, Y.L., 2007. Interactions between U-937 human macrophages and poly(propyleneimine) dendrimers. J. Control. Release 120, 51–59.

Kuo, J.H., Liou, M.J., Chiu, H.C., 2010. Evaluating the gene-expression profiles of HeLa cancer cells treated with activated and nonactivated poly(amidoamine) dendrimers, and their DNA complexes. Mol. Pharm. 7, 805–814.

Lee, J.H., Cha, K.E., Kim, M.S., et al., 2009. Nanosized polyamidoamine (PAMAM) dendrimer-induced apoptosis mediated by mitochondrial dysfunction. Toxicol. Lett. 190, 202–207.

Li, C., Liu, H., Sun, Y., et al., 2009. PAMAM nanoparticles promote acute lung injury by inducing autophagic cell death through the Akt-TSC2-mTOR signaling pathway. J. Mol. Cell Biol. 1, 37–45.

Lin, C.W., Jan, M.S., Kuo, J.H., et al., 2012. Protective role of autophagy in branched polyethylenimine (25K)- and poly(L-lysine) (30–70K)- induced cell death. Eur. J. Pharm. Sci. 47, 865–874.

Lv, H., Zhang, S., Wang, B., et al., 2006. Toxicity of cationic lipids and cationic polymers in gene delivery. J. Control. Release 114, 100–109.

Mannisto, M., Vanderkerken, S., Toncheva, V., et al., 2002. Structure–activity relationships of poly(L-lysines): effects of pegylation and molecular shape on physicochemical and biological properties in gene delivery. J. Control. Release 83, 169–182.

Martin, T.M., Plautz, S.A., Pannier, A.K., 2013. Network analysis of endogenous gene expression profiles after polyethyleneimine-mediated DNA delivery. J. Gene. Med. 15, 142–154.

Merkel, O.M., Beyerle, A., Beckmann, B.M., et al., 2011. Polymer-related off-target effects in non-viral siRNA delivery. Biomaterials 32, 2388–2398.
Mizushima, N., 2007. Autophagy: process and function. Genes Dev. 21, 2861–2873.
Mizushima, N., Yoshimori, T., Levine, B., 2010. Methods in mammalian autophagy research. Cell 140, 313–326.
Morgan, D.M., Larvin, V.L., Pearson, J.L., 1989. Biochemical characterization of polycation-induced cytotoxicity to human vascular endothelial cells. J. Cell Sci. 94, 553–559.
Morille, M., Passirani, C., Vonarbourg, A., et al., 2008. Progress in developing cationic vectors for non-viral systemic gene therapy against cancer. Biomaterials 29, 3477–3496.
Mukhopadhyay, S., Panda, P.K., Sinha, N., et al., 2014. Autophagy and apoptosis: where do they meet? Apoptosis 19, 555–566.
Navarro, G., de ILarduya, C.T., 2009. Activated and non-activated PAMAM dendrimers for gene delivery *in vitro* and *in vivo*. Nanomedicine 5, 287–297.
Omidi, Y., Hollins, A.J., Drayton, R.M., et al., 2005. Polypropylenimine dendrimer-induced gene expression changes: the effect of complexation with DNA, dendrimer generation and cell type. J. Drug Target. 13, 431–443.
Regnström, K., Ragnarsson, E.G., Köping-Höggård, M., et al., 2003. PEI—a potent, but not harmless, mucosal immuno-stimulator of mixed T-helper cell response and FasL-mediated cell death in mice. Gene Ther. 10, 1575–1583.
Regnström, K., Ragnarsson, E.G., Fryknäs, M., et al., 2006. Gene expression profiles in mouse lung tissue after administration of two cationic polymers used for nonviral gene delivery. Pharm. Res. 23, 475–482.
Roberts, R., Al-Jamal, W.T., Whelband, M., et al., 2013. Autophagy and formation of tubulovesicular autophagosomes provide a barrier against nonviral gene delivery. Autophagy 9, 667–682.
Shcharbin, D., Pedziwiatr, E., Blasiak, J., et al., 2010. How to study dendriplexes II: transfection and cytotoxicity. J. Control. Release 141, 110–127.
Wang, Y., Zheng, M., Meng, F., et al., 2011. Branched polyethylenimine derivatives with reductively cleavable periphery for safe and efficient *in vitro* gene transfer. Biomacromolecules 12, 1032–1040.

CHAPTER

7

The Role of Autophagy in Cell Death

Shensi Shen and Patrice Codogno

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Abstract

Autophagy is an evolutionarily conserved process responsible for protein degradation and organelle turnover. The literal translation of "autophagy" in Greek, "self-eating," inclines that autophagy may be a phenomenon of cellular suicide. Indeed, cells undergoing cell death often display marked autophagic features, leading to the coining of the term "autophagic cell death." However, linking autophagy to cell death is largely circumstantial, and whether it is the primary cause of cell death is intensively under debate. In contrast, autophagy, as a cellular recycling process, is an important protective mechanism against a range of various stress conditions including starvation. Therefore, the dialogue between autophagy and cell death programs influences the physiological homeostasis of cells and tissues. The imbalance between the two pathways is largely related to different human diseases, including cancer. Due to increasing interests in targeting autophagy pathway for different human diseases, molecular mechanisms of the cross talk between autophagy and cell death pathways have achieved major prominence. This chapter aims at summarizing the recent advances on the role of autophagy in cell death.

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INTRODUCTION

The term "autophagy" originates from the Greek word "phagy" inferring "eat," and "auto" meaning "self." Autophagy is an evolutionarily conserved phenomenon in eukaryotes by which the cytoplasmic components, including long-lived proteins and damaged organelles, are sequestered inside double-membrane vesicles and are delivered to lysosomes for degradation. Four decades ago, Novikoff and Clark observed "dense bodies" containing mitochondria from mouse kidneys or rat hepatocytes were shown to include lysosomal enzymes. Subsequently, in 1963, at the Ciba Foundation symposium, de Duve for the first time founded the field with the name "autophagy" to describe the double-membrane "dense bodies" that contain components of cytoplasm and organelles in various states of disintegration. de Duve suggested that most, if not all, living eukaryotic cells engage autophagic processes for nonspecific bulk digestion of their own mass in the lysosomes, and dwindle nutrient resources for cellular adaptation under various stress conditions. In addition to this well-studied role of autophagy in cellular homeostasis and survival, the function of autophagy in cell death has long been recognized. During 1960s and 1970s, cellular ultrastructure studies demonstrated that in *Drosophila* autophagic vesicles accumulate during early stages in the destruction of larval tissues. Although Lockshin and Williams coined the term "programmed cell death" for the first time early in the 1960s, until later in 1990s, Clarke outlined three major cell death morphologies during embryonic development, in which he classified autophagic cell death (ACD) as the Type II cell death with the appearance of double-membrane enclosed vesicles in the cytoplasm. However, this early classification of cell death solely based on morphological criteria is now questionable and is continuously refining. With the evolving knowledge on cell death subroutines, the Nomenclature Committee on Cell Death has classified cell death into five relatively well-documented modes, including intrinsic apoptosis, extrinsic apoptosis, regulated necrosis, mitotic catastrophe, and ACD. The net effect of autophagy on cell death is not limited to "ACD," however, it is highly contextual and its cross talk with other cell death modes has also been reported, revealing autophagy function as both cytoprotective and cytotoxic mechanisms. Given the fact that dysfunctional autophagy as well as excessive autophagy can have detrimental consequences on cell death outcomes, abrogation or activation of autophagy may both represent important strategies for treatment of different diseases. Therefore, the comprehensive understanding of the functional role of autophagy in cell death is essential for the future design of therapeutic treatments targeting this pathway.

In this chapter, we will at first give a short revisit the morphological and molecular basis of different cell death subroutines, document in detail what is known so far for the functional role of autophagy in different cell death subroutines. As a dynamic process, systematic and quantitative understanding how autophagy reconciles its survival and catabolic-related role with its pro-death property is a crucial quest in the field. We will also discuss this important issue in a perspective way in light of recent advances in quantitative biology and systems biology.

CELL DEATH SUBROUTINES

Programmed cell death, founded by landmark work from Lockshin and Williams in 1964, is an evolutionarily conserved mechanism that is crucial for cellular vital functions, including development, tissue homeostasis, and pathogen defenses. In 1972, Currie and Kerr introduced two major types of cell death: apoptosis, genetically programmed cell death, and necrosis, passive nonselective cell death. In the following several decades, apoptosis was recognized as a general term of programmed cell death, even so dominant that early observations about existence of other nonapoptotic programmed cell death were largely ignored. Until recently, the emerging knowledge on the molecular mechanisms of different types of programmed cell death now enables the classification into distinct "cell death subroutines," including (1) intrinsic apoptosis, (2) extrinsic apoptosis, (3) programmed necrosis, (4) mitotic catastrophe, and (5) ACD.

Apoptosis is morphologically characterized by cell shrinkage, DNA fragmentation (karyorrhexis), nuclear condensation (pyknosis), as well as plasma membrane blebbing that lead to formation of apoptotic bodies. Apoptosis is also biochemically defined by phosphatidylserine exposure and activation of effector caspases. Mitochondria have a pivotal role in intrinsic apoptosis, which is activated by intracellular stress cues. Activated Bak and Bax proteins (pro-apoptotic BCL-2 family members) bind to mitochondrial membrane and form transmembrane pores, leading to mitochondrial outer membrane permeabilization (MOMP) and release of pro-apoptotic factors, including cytochrome c, apoptosis-inducing factor (AIF), and endonuclease G (Endo G). In extrinsic apoptosis, extracellular death signals activate transmembrane receptors culminating in a caspase-dependent, apoptotic cell death. These two types of apoptotic cell death are not mutually exclusive, intensive cross talk between intrinsic and extrinsic apoptosis exists at multiple molecular levels.

In contrast to intensive studies on apoptosis, programmed necrosis is only recently recognized in the cell death community. Necrosis has been traditionally considered as a passive, nonprogrammed form of cell death, which is a result of bioenergetic deprivation from adenosine triphosphate (ATP), and was thought to be initiated by massive cellular injury. Necrosis is morphologically defined by cytoplasmic vacuolization, plasma membrane permeabilization, and inflammation. Programmed necrosis involves activation of the kinases receptor-interacting protein 1 (RIPK1) and RIPK3 and this process can be specifically blocked by RIP1 inhibitor necrostatin-1. The term "necroptosis" is specifically referred to necrostatin-1 inhibitable forms of programmed necrosis. It is still not well understood the molecular mechanisms for the execution of the programmed necrosis. However, this may involve energy failure, oxidative stress, and lysosomal membrane permeabilization.

Mitotic catastrophe is generally referred to cell death induced by a combination of deficient cell-cycle checkpoints and cellular damage, occurring during mitosis. However, mitotic catastrophe can exhibit both features of apoptosis and necrosis. Cell death occurring during the metaphase/anaphase transition is mediated by caspase-2 and/or mitochondrial membrane permeabilization. Cells that fail to execute a cell death program in response to mitotic failure will divide asymmetrically with consequently generation of aneuploid cells.

Since mitotic catastrophe exhibit morphologically features of apoptosis or necrosis, this classification as a stand-alone cell death mode is not widely accepted.

ACD exclusively restricts its use to cell death by autophagy, in which selective suppression of autophagy flux abrogates cell death and the execution of cell death is mediated by overactivated autophagy flux rather than apoptosis or necrosis. ACD is defined morphologically by massive autophagosomes in the cytoplasm. However, the mere presence of autophagosomes in dying cells could not be interpreted as a sign of ACD. In many descriptive cases of ACD consequently reflect cell death accompanied with autophagy (often because of blockage of autophagic flux). Therefore, cases of cell death that exhibit an increase of autophagosomes, the lipidation of LC3, or increased degradation of autophagic substrate p62, while cannot be suppressed by autophagy flux inhibition, should not be defined as ACD. Most convincing cases of *bona fide* ACD have been observed in model organisms, including nematodes and fruitfly *Drosophila melanogaster*. During *Drosophila* obsolete larval development, characteristics of autophagy and cell death have been shown pivotal for the development of the salivary glands and the midgut. Genetic inhibition of autophagy by mutations in *Atg2* or *Atg18*, or knockdown of either *Atg1* or *Atg18*, delays larval programmed cell death. Another model system, *Dictyostelium* brings considerable advantages to the study of ACD. *Dictyostelium* is a eukaryote, a protist, normally found in the soil of deciduous forests. It has no apoptosis machinery, such as caspase family members and BCL-2 family members, which could interfere with nonapoptotic ACD. It was reported by Tresse et al. (2008) that autophagy and ACD can be induced separately in *Dictyostelium*, dissociated into a starvation-induced sensitization stage, during which autophagy is induced and not sufficient to lead to ACD, and a ACD induction stage following differentiation into stalk cells. On the other hand, there are an even increasing number of studies showing true ACD in mammalian cells, especially in pathological or drug-induced cell loss. Nevertheless, given the double-edged function of autophagy with both protective and cytocidal mechanism, it is under intensive studies whether autophagy even under conditions of ACD may elicit complex cellular response beyond the modulation of cell death. In the following paragraphs, we will discuss in detail the functional role of autophagy in different cell death modes.

AUTOPHAGY AND APOPTOSIS

The most intensive study on the functional role of autophagy in cell death is its cross talk with apoptosis. The two distinct self-destructive mechanisms, autophagy ("self-eating") and apoptosis ("self-killing"), determine the final fate of cytoplasmic organelles and entire cells. Autophagy and apoptosis are often activated in the same cell, though frequently in a sequential activation in which autophagy precedes apoptosis. In many cases, autophagy functions primarily as an adaptive mechanism to maintain nutrient and energy homeostasis during stress conditions. Apoptosis is activated when overwhelmed stress exceeds critical threshold, and autophagy can be inactivated, in part due to caspase-mediated inactivation of key autophagy proteins. Besides this general scenario, autophagy may promote cellular demise, partially by degradation of essential portions of cells or favoring activation of apoptotic cell death pathways.

Autophagy Inhibits Apoptosis

Autophagy, as a degradation mechanism, can selectively target pro-apoptotic proteins or signaling molecules for lysosomal degradation and leads to shutting off apoptotic signaling pathways. It was reported by Boya et al. (2005) that autophagy is cytoprotective under conditions of nutrient depletion. The formation of autophagic vacuoles was enhanced at a preapoptotic stage, which accelerates apoptosis upon genetic inhibition of autophagy-related proteins or inhibition of lysosome and autophagosome fusion. Ubiquitylated proteins in the cytosol can be recognized by autophagy adaptor proteins, such as sequestosome 1 (SQSTM1 or p62), followed by binding with LC3 proteins for engulfment by autophagosomes. It was reported by Hou et al. (2010) that in Bax knockout colon cancer cell line HCT116 cells, TRAIL (TNF-related apoptosis-inducing ligand)-induced autophagy counter-reacts TRAIL-mediated apoptosis by continuous sequestration of the large caspase-8 subunit in autophagosomes and its subsequent degradation in lysosomes. Thus, HCT116 $Bax^{-/-}$ cells are resistant to TRAIL-mediated cell death unless autophagy is inhibited. This regulation has also been observed in a model of tumor necrosis factor (TNF)-induced hepatocyte apoptosis. It was reported by Amir et al. (2013) that liver-conditional knockout of *Atg7* largely increases caspase activity in hepatocytes. In addition, autophagy is also required for inhibition of anoikis induced by disrupted signaling of focal adhesion kinase (FAK). Detachment from extracellular matrix activates FAK-SRC signaling pathway-mediated apoptosis. It was reported by Sandilands et al. (2012) that activated SRC can bind to E3 ubiquitin protein ligase CBL, which is an adaptor protein containing LC3-interacting region, leading to autophagic elimination in the lysosomes. Interestingly, autophagic adaptors, such as SQSTM1, can also function as pro-apoptotic signaling molecules through stimulating cytosolic reactive oxygen species. Autophagy decreases the abundance of SQSTM1 to inhibit cell death.

Apart from targeting specific signaling molecules, extensive studies have shown that selective removal of dysfunctional organelles by autophagy reduces the tendency of cells to undergo apoptosis. Mitochondrion is the central organelle where survival and death signals converge to determine whether or not intrinsic apoptosis is activated. Different regulators, including protein kinases, lipids, and metabolites, affect the integrity of the mitochondrial membranes. During cell death, MOMP initiates the release of mitochondria localized factors, including caspase activators (such as cytochrome c and Smac (also known as Diablo)) and catabolic hydrolases (such as AIF and Endo G). Meanwhile, it causes inner mitochondrial transmembrane potential ($\Delta\psi_m$) to dissipate, leading to bioenergetic catastrophe. Autophagy can degrade damaged mitochondria and inhibits apoptosis through increasing the threshold for cell death induction.

At the molecular level, it was reported by Okatsu et al. (2012) that loss of $\Delta\psi_m$ or permeabilization of the inner membrane inhibits a protease that cleaves the PTEN-induced putative kinase 1 (PINK1), which causes accumulation of PINK1 at the surface of damaged mitochondria. Accumulated PINK1 then recruits and phosphorylates Parkinson's disease protein (PARKIN). PARKIN is an E3 ubiquitin ligase that leads to Lys63 ubiquitylation of outer mitochondrial membrane proteins, including voltage-dependent anion-selective channel 1 (VDAC1), mitofusin1 (MFN1), and MFN2. The ubiquitylated proteins function as tags of the damaged mitochondria for degradation by mitophagy. In addition to $\Delta\psi_m$ dissipation,

mitochondrial fragmentation through fission is also required for mitophagy induction. Indeed, mitochondrial morphology changes during apoptosis, resulting in small, round organelles, which occurs early in the apoptotic cell death. Dynamin-related protein 1 (DRP1) and endophilin B1 translocate from cytosol to foci on mitochondria early during apoptosis and they mediate apoptotic mitochondrial fission. Fragmented mitochondria are then engulfed by phagophores to form mitophagosomes followed by degradation in lysosomes. Interestingly, mitochondria in starved cells undergoing $\Delta\psi_m$ dissipation but not fragment do not experience mitophagy, this indicates that both $\Delta\psi_m$ dissipation and mitochondria fission function together as fail-safe mechanisms to avoid unwanted removal of functional mitochondria during mitophagy.

Autophagy can also inhibit other organelle-initiated apoptosis through autophagic removal of the damaged organelles. For example, in pancreatic acinar cells, different enzyme precursors (zymogens), such as trypsinogen (the precusor of trypsin), are sequestered and inactivated in cytoplasmic granules. However, these toxic zymogens can be released into cytosol under stress conditions and cause cell death. It was reported by Grasso et al. (2011) that autophagy is induced upon autophagy-promoting protein vacuole membrane protein 1 (VMP1) overexpression in a model of cerulein-mediated pancreatitis. VMP1-induced autophagy engulfs cytoplasmic granules containing aberrantly activated trypsin and prevents cell death in pancreatic acinar cells. In contrast, blockage of autophagic flux exacerbates pancreatitis induced by ethanol and bacterial lipopolysaccharide (LPS).

Autophagy Promotes Apoptosis

Autophagy is a dynamic process that initiates phagophore extension and closure to form autophagosome and degrades its engulfed components through fusion with lysosome. Autophagosome, as a cytoplasmic organelle, can function as a platform for the activation of different pro-cell death signaling molecules. Moreover, several essential proteins involved in autophagy pathway may have additional roles in pro-apoptosis signaling. To be noted, some autophagy-related genes are not exclusively involved in autophagy in this context, rather they are also implicated in different other cellular processes, such as endocytosis and protein secretion. Thus, when we are studying the functional role of autophagy in cell death, it is important to distinguish the roles of genes or proteins from the other processes.

Indeed, it was reported by Espert et al. (2006) that HIV-infected cells can induce autophagy in bystander $CD4^+$ T lymphocytes through contact of HIV-1 envelope glycoproteins with CXCR4 (chemokine receptor type 4), leading to apoptotic cell death and contributing to immunodeficiency. Autophagosome has later been shown to be an activated platform to promote apoptosis signaling. In this context, it was reported by Young et al. (2012) that in SV40-large T antigen-immortalized mouse embryonic fibroblasts (MEFs), treatment of SKI-1, a pan-sphigosine kinase inhibitor, and bortezomib, a proteasome inhibitor, can induce an autophagy-dependent apoptotic response. Caspase-8 forms a complex with Atg5 and colocalizes with LC3 and SQSTM1, in which FADD, an adaptor protein for caspase-8 activation, associates with Atg5 on Atg16L- and LC3-positive autophagosomal membranes. Autophagosome formation thus induces concomitantly with intracellular death-inducing signaling complex (iDISC) and caspase-8 activation, which is responsible for activation of caspase cascade and full execution of apoptosis. As discussed above,

autophagy can inhibit apoptosis through sequestering caspase-8 in the autophagosome followed by lysosomal degradation. How the iDISC complex hijacks autophagosomes for activation of caspase cascade, and what upstream signaling cues determine whether autophagy can activate or inhibit caspase-8 are remained to be elusive.

In addition to directly participating pro-apoptotic signaling activation, autophagy has been implicated to degrade apoptotic inhibitory signal molecules to promote cell death. It was reported by Gump et al. (2013) that selective autophagic degradation of the Fas-associated phosphatase 1 (FAP-1, also known as PTPN13) promotes Fas-mediated apoptosis in Type I cells (which do not require mitochondrial permeabilization for efficient apoptosis). FAP-1 specifically dephosphorylates Fas receptor and reduces its cell surface expression and activity. FAP-1 interacts with SQSTM1 in a Fas-ligand-dependent manner, and this interaction is further strengthened with autophagy flux inhibition by chloroquine treatment. This indicates that SQSTM1 associates directly with FAP-1 enabling its autophagic degradation in response to Fas receptor activation and regulates the apoptotic response in Type I cells. Accordingly, overexpression of FAP-1 causes Type II cells to behave like Type I cells in response to autophagy inhibition and Fas ligand. Conversely, *Fap1* knockdown abrogated the increase in cell death caused by chloroquine in response to Fas ligand in Type I cells. Autophagy thus promotes apoptosis in a stimuli- and cell-type-specific manner.

Autophagy promoting apoptosis by depleting inhibitors of apoptotic pathways has also been observed in model organisms. In *D. melanogaster*, it was reported by Nezis et al. (2010) that autophagy controls the DNA fragmentation during late oogenesis. The *Drosophila* inhibitor of apoptosis (IAP) dBruce (BIR-containing ubiquitin-conjugating enzyme) was found to colocalize with autophagic marker GFP-Atg8a and be degraded by autophagy. Inhibition of autophagy flux by genetic depletion of the autophagy-related genes *Atg1*, *Atg13*, and *Vps34* results in late stage egg chambers that contain persisting nurse cell nuclei without fragmented DNA and attenuation of caspase-3 activation. Therefore, autophagic degradation of the IAP protein controls the interplay between caspases and IAPs, and triggers apoptosis in nurse cells.

Apoptosis Inhibits Autophagy

Although autophagy actively regulates apoptosis signaling pathways in a context-dependent manner, evidences have been shown that apoptotic signaling pathway can modulate autophagy activity or even hijack autophagy-related proteins to promote self-activation. Both intrinsic and extrinsic apoptosis are often accompanied with a high level of caspase activation, which results in cleavage of caspase substrates and leads to apoptotic morphologies. However, caspase activation has also been shown to inactivate essential autophagy-related proteins to attenuate cytoprotective function of autophagy and to accelerate cell demise. Caspase-8 and caspase-3 have been shown to digest autophagy-related proteins Atg3 and Beclin 1 (BECN1), the cleavage of which leads to the loss of their autophagy-stimulatory function. In the similar way, caspase-3 and calpains irreversibly degrade autophagy-related protein AMBRA1 (activating molecule in BECN1-regulated autophagy 1) to inhibit autophagy activation in Jurkat cells. The digestion of autophagy-related proteins by apoptotic caspases attenuates the threshold of cell death activation limited by adaptive stress-mediated autophagy and leads to apoptosis activation.

Caspase cleavage, in some contexts, even can hijack and modify autophagy-related proteins to acquire pro-apoptotic functions. Indeed, it was reported by Wirawan et al. (2010) that Beclin 1 is cleaved by caspase-3, caspase-6, and caspase-9, resulting in the generation of a carboxy-terminal fragment of Beclin 1. This cleaved form of Beclin 1 then localizes to mitochondria *in vivo* and permeabilizes mitochondria *in vitro* to activate cytochrome c releasing. Similarly, autophagy-related protein Atg4D turns out to be pro-apoptotic following its cleavage by caspase-3. In addition to caspase-mediated cleavage, it was reported by Yousefi et al. (2006) that calpains mediate the cleavage of Atg5, resulting in the generation of an amino-terminal fragment 24KDa form of Atg5. The cleaved form of Atg5 sensitizes cells to CD95-, ceramide-, and anticancer drug- (etoposide and doxorubicin) mediated apoptosis, in spite of the cell types. Consistently, calpain-mediated Atg5 cleavage also switches protective autophagy induced by sphingosine-1-phosphate phosphohydrolase 1 deficiency to apoptosis activation.

Despite the fact that autophagy and apoptosis proceed through independent mechanisms, there are extensive cross talk between the two pathways. Cellular stress-responses to the same stimuli may manifest predominantly by autophagic or apoptotic mechanism depending on cellular context. On the other hand, autophagy and apoptosis might depend on each other in some cellular context, thus abrogation of one might influence the progress of the other. These two "self-destructive" mechanisms are both dynamically regulated in the same cells, therefore, the understanding of the functional role of autophagy in apoptosis and their cross talk will depend on single-cell dynamic analysis and extensible identification of functional motifs at the systems level in the future. We will discuss this point of view in later part of this chapter.

AUTOPHAGY AND PROGRAMMED NECROSIS

As mentioned above, necrosis has been seen as an unregulated accidental cell death for many years. However, with the evolution of genetic, biochemical evidence and the discovery of specific chemical inhibitors of necrosis, programmed necrosis has come to age. To dates, programmed necrosis includes several cell death modalities, among which necroptosis and pyroptosis are the best characterized forms of programmed necrosis. Necroptosis is a form of caspase-independent cell death depending on the activation of receptor-interacting protein kinase-1 (RIPK1) and 3 (RIPK3) and its substrate mixed lineage kinase like (MLKL). Necroptosis can be activated by cell surface receptors, notably including TNF family death receptors and toll-like receptors (TLRs). Pyroptosis is an inflammatory form of cell death mediated by inflammasome activation and subsequent caspase-1 and caspase-11 activation. In contrast to apoptosis, these programmed necrosis result in the release of pathogen-associated molecular patterns or damage-associated molecular patterns, and cytokines that activate pro-inflammatory immune cell response. Intriguingly, necroptosis and pyroptosis are often accompanied by double-membrane autophagosomes in the cytosol. We will discuss autophagy process or autophagy-related proteins involved in programmed necrosis by focusing on necroptosis and pyroptosis.

In 2005, it was reported by Degterev et al. (2005) that they identified for the first time necroptosis mediated by Fas/TNFR receptor family in the absence of intracellular apoptotic signaling, which is characterized by necrotic cell death morphology and activation

of autophagy. A specific chemical inhibitor, termed necrostatin-1 (Nec-1), can block both necroptosis and autophagy, indicating the potential cross talk between autophagy and necroptosis. Indeed, autophagic membranes can recapitulate the protein complexes required for necroptosis and with other modulators to keep necroptosis in check. In human T cells, it was reported by Bell et al. (2008) that autophagy recruits caspase-8 and its paralog $cFLIP_L$ through interaction with FADD-Atg5-Atg12 complexes, forming a feedback loop to limit autophagy. Upon lacking FADD or caspase-8, autophagy induction in T cells switches cell survival to a RIPK1-dependent necroptosis, which is completely rescued by Nec-1. Autophagy induction has also been observed in rhabdomyosarcoma cells treated with the pan-Bcl-2 family inhibitor Obatoclax. It was reported by Basit et al. (2013) that this inhibitor triggers the interaction of Atg5 with components of the necroptotic signaling pathway such as FADD, RIPK1, and RIPK3 on the autophagosome membranes, leading to potent RIPK3 activation and necroptotic cell death. Blockade of autophagosome formation by silencing *Atg5* or *Atg7* abolishes Obatoclax-induced necroptosis, indicating an autophagy-dependent necroptotic mechanism. Therefore, whether autophagy promotes or inhibits necroptosis depends on the stimuli and cellular transcriptional signature. Both TNF-family death receptors and TLRs induce autophagy and activate NFκB pathways, leading to increase of necroptosis inhibitory molecules such as caspase-8 and $cFLIP_L$. Recruitment of caspase-8 or $cFLIP_L$ to autophagosome-dependent RIPK necrosome complex will keep necroptosis activation in check. However, drug-mediated autophagy activation, lacking general transcriptional regulation of necroptosis inhibitory signatures, leads to potent RIPK3 activation and subsequent necroptosis in cells.

Pyroptosis is triggered in inflammatory cells in response to excessive inflammation by inflammasome activation and caspase-1-dependent release of proinflammatory cytokines, such as IL-1β, IL-18, and IL-33. Cytosolic receptors of the Nod-like receptor family, including NLRP3 and NLRP1, interact with adaptor proteins to form inflammasome complexes. The NLRP3 interacts with apoptosis-associated speck like protein containing CARD and activates procaspase-1 by proteolytic cleavage. In macrophages, autophagy has been shown to delay the onset of pyroptosis by degradation of inflammasome components in response to infections. Indeed, it was reported by Shi et al. (2012) that activation of inflammasome pathways induces autophagosome formation through activation of the GTPase RalB, forming a negative feedback checkpoint to avoid excessive inflammasome activation by turnover and degradation of ubiquitinated inflammasome complexes. The inhibitory function of autophagy in pyroptosis is closely related to tissue-level inflammation response. It was reported by Saitoh et al. (2008) in another study that the proinflammatory cytokine secretion (e.g., IL-1β) following pyroptosis was enhanced in *Atg16L* or *Atg7* knockout macrophages in response to LPS, which was validated *in vivo* that mice lacking *Atg16L* displayed increased susceptibility to a murine model of colitis. Although there is no direct evidence that autophagy facilitates pyroptosis progress, a recent study demonstrates that inflammasome pathway can take advantage of autophagy pathway to enhance cytokine secretion. Autophagy induced by starvation can enhance inflammasome-mediated caspase-1 activation and secretion of IL-1β and IL-18 in an unconventional autophagy-dependent secretion pathway. Therefore, current knowledge about the function of autophagy in necroptosis and pyroptosis warrants further investigation in models of inflammatory cell death and its relationship with tissue-level immune response.

AUTOPHAGY AND ACD

We have seen intensive regulatory function of autophagy in different cell death modalities as described above, and in most settings, autophagy appears to serve as a pro-survival stress response mechanism. However, increasing evidence for autophagy *per se* mediating cell death, termed ACD, has been shown in the last decade. Autophagy activity is kept in strict check at basal level, whereas when autophagic activity overactivates above a certain threshold, it can destroy a major portion of the cytosol and organelles, leading to an irreversible type of cellular atrophy and collapse of cellular functions. Indeed, during extensive autophagy, the total area of autophagic vacuoles may be roughly equal to, even greater than, that of cytosol and organelles outside the vacuoles. Therefore, overwhelmed autophagy in cells could lead to cellular demise. It should be noted that so-called ACD is strictly restricted to that final cell death process is mediated by enhanced autophagy flux rather than by other cell death modalities, such as apoptosis or necroptosis. As we have mentioned above, much of the evidence in favor of true ACD has been produced in model organisms, including *Caenorhabditis elegans* and *D. melanogaster*. Autophagy inhibition can indeed prevent the manifestation of autophagic programs manifesting in terminally differentiated cells, and delay their disappearance during developmental programmed cell death, suggesting that ACD plays a role in invertebrate development. In addition, ACD has also been shown in mammalian cells, we outline some examples below.

Autophagy-related gene Beclin 1 is at the center of the regulation of autophagy flux in mammalian cells, it was reported by Liu et al. (2013) in a recent study that an autophagy-dependent cell death termed autosis by targeting Beclin 1 complex through a cell-permeable autophagy-inducing peptide (Tat-BECN1). Indeed, Tat-BECN1 induced a time- and dose-dependent cell death and genetic or pharmacological inhibition of autophagy, but not of apoptosis or necroptosis, can abrogate autotic cell death. Meanwhile, the scientists found that antagonists of the ion pump Na^+/K^+ ATPase or genetic knockdown of the Na^+/K^+ ATPase α1 subunit can also completely block autosis. This indicates that autosis does not overlap genetically with apoptosis and necroptosis, in addition, this type of ACD is characterized by enhanced cell–substrate adherence, dilated and fragmented endoplasmic reticulum, nuclear membrane convolution, and focal swelling of the perinuclear space. Therefore it displayed unique morphological features. Apart from peptide-mediated autosis, it has also been observed in pathological conditions, such as hippocampal rat neurons during cerebral ischemia. Therefore, high amounts of the BH3-only protein Beclin 1 can overactivate autophagy flux to trigger ACD in the absence of other cellular stresses. In a similar way, it was reported by Zhaorigetu et al. (2008) that overexpression of the BH3-only protein apolipoprotein L1 has shown to induce ACD.

In cell malignancy, ACD is strictly regulated, serving as a failsafe mechanism to surveil malignant transformation. Acute oncogenic stress potentiates autophagy activity to induce ACD as a safeguard mechanism to limit the oncogenic transformation in the early stage of deregulated oncogene mutation. Indeed, it was reported by Elgendy et al. (2011) that the oncogene H-Ras triggers upregulation of BH3-only proteins Noxa and Beclin 1 and a caspase-independent ACD in ovarian surface epithelial cells. Depletion of *Atg5*, *Atg7*, or Beclin can prevent cell death and restore the clonogenicity of cells that would die following acute overexpression of oncogenic RAS. An intrinsic form of ACD was later identified in multiple

myeloma in an RNAi-based screening. It was reported by Lamy et al. (2013) that caspase-10 was found to be required for multiple myeloma survival, irrespective of their genetic backgrounds. Caspase-10 thus inhibits overactivated autophagy by cleaving the Bcl2-interacting protein BCLAF1, which is a strong autophagy inducer by displacing Beclin 1 from its inhibitory protein Bcl-2. Therefore, in multiple myeloma cells, caspase-10 tempers the autophagy flux to fulfill cellular energy requirement and avoid cell death.

Moreover, there is evidence indicating that a selective destruction of certain cellular components, which could contribute to cell death mediated by autophagy. Catalase is one of the examples of the selective autophagy target contributing to cellular demise. It was reported by Yu et al. (2006) that zVAD-induced autophagy in several cell types selectively degrades catalase, a key enzyme of the cellular antioxidant defense mechanism. Furthermore, in melanoma cells, excessive mitochondria clearance by mitophagy leads to ACD. It was reported by Wang et al. (2014) that TR3-targeting compound 1-(3,4,5-trihydroxyphenyl)nonan-1-one triggers the orphan nuclear receptor TR3 translocation to the mitochondrial inner membrane through Tom40 and Tom70 channel proteins. This leads to mitochondrial membrane potential dissipation and induction of mitophagy, in turn executing irreversible mitophagy-dependent cell death. Lethal mitophagy has also been observed in C_{18} ceramide-induced growth inhibition. C_{18} ceramide causes DRP1-mediated mitochondrial fission. Knockdown of LC3 can reduce the susceptibility of tumor cells to C_{18} ceramide-induced cell death, as DRP1 incorporates into mitochondrial membranes and directly interacts with LC3, thereby leading to mitophagy-dependent cell death.

We have seen explosive increase of the researches on autophagy functional role in regulation of cell death in the past decades. Indeed, autophagy signaling pathway is now at the forefront of cancer therapy, with approximately 20 ongoing clinical trials in multiple varied cancers employing modulation of autophagy as either monotherapy or in combination with other traditional chemotherapies. However, the complex interaction between autophagy and other cell death modalities at molecular level leads to the net effect of autophagy on cell death highly contextual. Both cytoprotective and cytocidal functions of autophagy have been observed in stimuli- and cell context-dependent manner, which leads to the outcome of autophagy-targeted strategy in the most cases, if not all, unpredictable. On the other hand, similar to apoptosis, autophagy is a highly dynamical process under strict check in physiological conditions to avoid undesirable overactivation. Better understanding how cellular network is rewired to control autophagy activity and its function in cell fate outcomes will require in the future not only systematic view of the regulation of autophagy, but also dynamic analysis of the autophagy network at single cell level. We will describe autophagy regulation in a perspective view at systematic and dynamic level in the following paragraphs.

AUTOPHAGY: A COMPLEX DYNAMICAL SYSTEM

In the last half of the twentieth century, the dominant experimental modality in cell death studies was hypothesis-directed research, following a general tradition in biology that the molecular insights are gained from directed poking around and following hunches. However, recent development in genomic biology and systems biology starts to reexamine

this prejudice. Large-scale multivariate data and quantitative systems-level analysis now enable systematic fishing expeditions—undirected fishing for information hidden in the complex biological networks. Moreover, this complex biological network is at the center for processing external or internal cues through interactions or allosteric modifications of its components. Signaling networks can be remarkably attuned to sensing and distinguishing subtle features of stimuli to process key decisions regarding cellular response and cell fate. Therefore, due to the complicate interactions between autophagy network and other cell death modalities that we have seen so far, systems-level perspective for the comprehensive analysis of the autophagy regulatory network will be essential for future understanding of the paradoxical role of autophagy in cell death and its application to clinical therapies. Here, we consider two possible correlated directions for improving our understanding the functional role of autophagy in cell death, including causal network analysis of autophagy regulatory circuitry and dynamical analysis of information processing taking into account of cell-to-cell variations at single cell level.

A signaling network may contain nodes (or proteins) with multiple functions and interaction edges, thus an individual node might affected by multiple edges, forming different network motifs. The cellular response, here specifically referred to autophagy-regulated cellular fate decision, is determined by conflicting or cooperating interactions between the components rather than the characteristics of each individual component within the network. Which proteins are involved in the autophagy network? And how these molecules within autophagy network are rewired? These questions are now in their infancy. Recent development of high-throughput, parallel technologies such as proteomic-based protein–protein interaction network, genome-scale RNA interference screening, and high-content cell image-based phenotypic screening provide biologists unprecedented tools to study the overall organization of the autophagy network. It was reported by Behrends et al. (2010) that proteomic analysis of autophagy network gave a global view of the mammalian autophagy interaction landscape. The researchers expressed 32 human Flag-hemagglutinin (HA)-tagged autophagy-related proteins into human 293T cells, and proteins in anti-HA immune interaction complexes were identified by high-throughput mass spectrometry. This proteomic-based protein interaction analysis revealed a network of 751 interactions among 409 autophagy-related candidate-interacting proteins with extensive connectivity among subnetworks, providing an insight into the complex organization of autophagy network and serving as a resource for mechanistic analysis. Similarly, it was reported by Lipinski et al. (2010) that a genome-scale RNA interference screening study has characterized a group of 236 genes involved in the regulation of physiological basal-level autophagy probing the homeostatic autophagy regulation in cell death. Development of computational analysis tools also favors the reconstruction of the network of autophagy, it was reported by Jegga et al. (2011) that they have developed a systems biology-based integrative analysis to study the molecular interactions in autophagy–lysosome pathways. Specifically, they have analyzed transcriptional and microRNA-based posttranscriptional regulation of autophagy-related genes with functional enrichment analyses to reveal their involvement in nervous system related diseases and phenotypes.

Although large-scale screening and protein–protein interaction landscape can identify potential network inter-connectivity involved in autophagy pathways, proper understanding of the cellular response and their potential disregulation requires multivariate

approaches to study how the network components are hierarchically organized, termed the causality. Bayesian network has been proffered as a promising framework for modeling complex systems because this statistical method can represent the probabilistic dependence among multiple interacting components. In addition, Bayesian network can accommodate biological and experimental noise in biologically derived data, describing direct molecular interactions as well as indirect influences that proceed through unobserved components. When combined with multivariate single cell data and independent perturbations, Bayesian network inference can take into account large samples from single cell data to accurately predict pathway hierarchical structure. This computational strategy has been applied to reverse engineering and inference of the basic structure of a signaling pathway in human T-cell differentiation reported by Sachs et al. (2005). A direct possible application of Bayesian network to autophagy studies is to infer autophagy-related gene network based on gene expression profiles upon autophagy specific inducer or inhibitor treatment. However, this data resource would be limited because the regulation of autophagy and cell death is largely dependent on protein modifications and interactions. An alternative way is using multivariate flow cytometry method to probe intracellular autophagy-related protein activity (e.g., phosphorylation) in individual cells, thus by collecting large samples of single cells, Bayesian network can treat each single cell as an independent event in the statistical space to infer the protein–protein hierarchical interactions even though currently we can only detect simultaneously approximately 14 different molecules in a single cell (e.g., limitation of the fluorophores availability). This limitation of low dimensions of molecular events detected by fluorophore-based flow cytometry could be overcome with development of the mass cytometry, which uses metal-chelated antibodies to detect intracellular protein activities arriving at the level of detecting approximately 40 molecules at the same time in a single cell. Therefore, based on our prior knowledge on the molecular mechanisms of the autophagy regulation in cell death, we can envision a vague advance in understanding the network organization of this complex signaling pathway with the help of novel parallel screening systems and computational systems-level strategies.

Another important aspect concerning the functional role of autophagy in cell death is its dynamic regulation and activation. Current knowledge on autophagy in cell death that we have described so far is largely qualitative and descriptive, and the complex circuits of autophagy that integrate intrinsic or extrinsic signals to execute its pro-survival and pro-death function are still poorly understood. Although large-scale multivariate experimentation combining with integrative computational analysis could shed light on the hierarchical structure of this signaling network, we still suffer to understanding how the cell temporally and spatially organizes its “self-destructive” pathways to determine its binary life-death decisions. In addition, the intrinsic stochastic nature of live cells and the “noisy” complex extrinsic microenvironment determine high cell-to-cell heterogeneity of the cellular response even in an isogenic clonal population, which might mislead our interpretation of the biological processes depending solely on population-level analysis. For example, the dynamic analysis of apoptosis at single cell level has revolutionized our recognition on the regulation and initiation of apoptosis. With the development of fluorescent reporters for MOMP and caspase substrate cleavage, the scientists have collected data on the dynamics of apoptosis in single cells. It was reported by Spencer et al. (2009) that following exposure to stimuli of apoptosis, for instance staurosporine or TNF-family death receptor ligands,

cells wait for several hours before initiating a set of rapid, all-or-none events that triggers MOMP and caspase activation. This novel single-cell dynamic analysis contradicts with data obtained earlier by Western blotting and other population-average biochemical assays that have shown that MOMP and caspase activation occur gradually over a period of time. With the aid of mathematical modeling of signaling pathways, the scientists are able to reconcile these two types of data by noting that apoptosis is sudden and switch like process in individual cells, but that it takes place heterogeneously at different times in different single cells. With this example as one among many, it follows then that to properly understand autophagy functional role in cell death and its disregulation in diseases, robust quantitative characterizations of signaling relationships in the autophagy network will be required to enable more accurate models of signaling.

Indeed, autophagy is a highly dynamic catabolic process, whose activity is sustained at a basal level under physiological conditions to support cellular energetic requirement. As we have seen in previous paragraphs, the apparent dichotomy between cytoprotective and cytotoxic autophagy might be causally correlated with the extent and duration of autophagy activity and its cross talk with other cell death modalities. Two different laboratories have performed pioneer works on studying the dynamics of autophagy by using GFP-LC3, a robust autophagosome marker. It was reported by Martin et al. (2013) that GFP-LC3 autophagosome accumulates gradually in cells after stimulation, similarly it was reported by Xu et al. (2013) that the researchers have found subsequent initiation of apoptosis accompanying shut off of autophagy activity when autophagy arrives at some threshold extent. Mathematical modeling of the ATG protein network reconstitutes the dynamic response of autophagy showing multiple feedback loops are required for the analog response of autophagy induction. We noted that most of the upstream autophagy regulatory proteins actually are transiently activated during autophagy induction within minutes, but how these pulse-like signals can be transformed into gradual increase of autophagy activity? To answer this kind of question, we will have to simultaneously monitor the activity of multiple nodes of the autophagy regulatory network in the same cell to decipher the transmission of the signals in this pathway. Certainly, this strategy will give us a chance to determine the threshold level of autophagy that dictating life-death decisions. It can also serve to analyze the signaling landscape of autophagy network to understand how the cells rewire the network dynamically to keep basal-level autophagy at steady state under physiological conditions.

Moreover, cell-to-cell autophagy variation seems also contribute to cell death regulation. Nongenetic heterogeneity, stochastic state differences, and variation have been seen in many cellular processes, such as apoptosis and differentiation. Particularly, for the question whether autophagy role in cell death is pro-survival or pro-death, we have to take into account the variability of autophagy between single cells. This is because even in the same population of cells, autophagy can be both cytoprotective and cytocidal due to its activity variation between individual cells. For example, a recent study has quantified cell death induced by TNF-family death receptors in different subpopulations with different autophagy activity. It was reported by Gump et al. (2013) that using an mRFP-GFP-LC3 fusion protein marker, the scientists have separated subsets of cells based on low, medium, and high activity of autophagy. Surprisingly, they have found that the cells with low autophagy activity demonstrate resistance against death receptor ligand interrogation.

However, the cells with high autophagy activity show high sensitivity to cell death inducers, in which high autophagy flux actually degrades extrinsic apoptosis-inhibitory phosphatase FAP-1. Thus, this work provides an example whereby stochastic variability of autophagy activity can determine cell fate. It is plausible to imagine that if we do not take into account the cell-to-cell heterogeneity of autophagy, it will be difficult to predict the outcome of a treatment that targets to autophagy pathway.

In our bodies, autophagy is happening all the time. Literally saying, our cells are eating themselves every second, but we are not dying. The tight regulation of the dynamics of autophagy dictates its functional role in determining cell survival, proliferation, and death. With the development of novel systems-level technologies, such as single-cell multiplexed imaging, single-cell high-throughput microfluidics, and high-performance computational analysis, we will unveil the whole image of autophagy role in cell death and maybe even reengineer the control of autophagy in live cells.

CONCLUSIONS

Autophagy has been discovered more than 40 years ago, at which time the scientists were perplexed as to why our cells self-digest its own components even under physiological conditions. After decades of studies on autophagy regulation and function in different diseases, we have since learnt that autophagy is not simply a cellular rubbish disposal mechanism, rather it involves in maintaining nutrient and energy homeostasis during stress conditions, participates in clearing misfolded proteins, damaged organelles, and invasive pathogens. In this chapter, we have tried to summarize an abstracted view of the functional role of autophagy in cell death. However, notably, what we have seen so far represents only the tip of the iceberg. Since it is often difficult to separate the independent role of autophagy in cell death during the rapid destruction, the precise functional role of autophagy during cell death is rather complicated. Comprehensive understanding of the physiological and pathological role of autophagy in cell death will be further advanced with the development of novel systems-level and quantitative methods that treat the autophagy network as a dynamical complex system. Nevertheless, a full understanding of the paradoxical role of autophagy in promoting cell survival and death will be essential for mechanistic assessment of autophagy role in cell death and its application in therapeutics.

References

Amir, M., Zhao, E., Fontana, L., et al., 2013. Inhibition of hepatocyte autophagy increases tumor necrosis factor-dependent liver injury by promoting caspase-8 activation. Cell Death Differ. 20, 878–887.

Basit, F., Cristofanon, S., Fulda, S., 2013. Obatoclax (GX15-070) triggers necroptosis by promoting the assembly of the necrosome on autophagosomal membranes. Cell Death Differ. 20, 1161–1173.

Behrends, C., Sowa, M.E., Gygi, S.P., et al., 2010. Network organization of the human autophagy system. Nature 466, 68–76.

Bell, B.D., Leverrier, S., Weist, B.M., et al., 2008. FADD and caspase-8 control the outcome of autophagic signaling in proliferating T cells. Proc. Natl. Acad. Sci. U.S.A. 105, 16677–16682.

Boya, P., Gonzalez-Polo, R.A., Casares, N., et al., 2005. Inhibition of macroautophagy triggers apoptosis. Mol. Cell. Biol. 25, 1025–1040.

Degterev, A., Huang, Z., Boyce, M., et al., 2005. Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat. Chem. Biol. 1, 112–119.
Elgendy, M., Sheridan, C., Brumatti, G., et al., 2011. Oncogenic Ras-induced expression of Noxa and Beclin-1 promotes autophagic cell death and limits clonogenic survival. Mol. Cell 42, 23–35.
Espert, L., Denizot, M., Grimaldi, M., et al., 2006. Autophagy is involved in T cell death after binding of HIV-1 envelope proteins to CXCR4. J. Clin. Invest. 116, 2161–2172.
Grasso, D., Ropolo, A., Lo, Re, A., et al., 2011. Zymophagy, a novel selective autophagy pathway mediated by VMP1-USP9x-p62, prevents pancreatic cell death. J. Biol. Chem. 286, 8308–8324.
Gump, J.M., Staskiewicz, L., Morgan, M.J., et al., 2013. Autophagy variation within a cell population determines cell fate through selective degradation of Fap-1. Nat. Cell Biol. 16, 47–54.
Hou, W., Han, J., Lu, C., et al., 2010. Autophagic degradation of active caspase-8: a crosstalk mechanism between autophagy and apoptosis. Autophagy 6, 891–900.
Jegga, A.G., Schneider, L., Ouyang, X., et al., 2011. Systems biology of the autophagy-lysosomal pathway. Autophagy 7, 477–489.
Lamy, L., Ngo, V.N., Emre, N.C., et al., 2013. Control of autophagic cell death by caspase-10 in multiple myeloma. Cancer Cell 23, 435–449.
Lipinski, M.M., Hoffman, G., Ng, A., et al., 2010. A genome-wide siRNA screen reveals multiple mTORC1 independent signaling pathways regulating autophagy under normal nutritional conditions. Dev. Cell 18, 1041–1052.
Liu, Y., Shoji-Kawata, S., Sumpter, R.M., et al., 2013. Autosis is a Na^+, K^+-ATPase-regulated form of cell death triggered by autophagy-inducing peptides, starvation, and hypoxia-ischemia. Proc. Natl. Acad. Sci. U.S.A. 110, 20364–20371.
Martin, K.R., Barua, D., Kauffman, A.L., et al., 2013. Computational model for autophagic vesicle dynamics in single cells. Autophagy 9, 74–92.
Nezis, I.P., Shravage, B.V., Sagona, A.P., et al., 2010. Autophagic degradation of dBruce controls DNA fragmentation in nurse cells during late *Drosophila melanogaster* oogenesis. J. Cell Biol. 190, 523–531.
Okatsu, K., Oka, T., Iguchi, M., et al., 2012. PINK1 autophosphorylation upon membrane potential dissipation is essential for Parkin recruitment to damaged mitochondria. Nat. Commun. 3, 1016.
Sachs, K., Perez, O., Pe'er, D., et al., 2005. Causal protein signaling networks derived from multiparameter single-cell data. Science 308, 523–529.
Saitoh, T., Fujita, N., Jang, M.H., et al., 2008. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. Nature 456, 264–268.
Sandilands, E., Serrels, B., Wilkinson, S., et al., 2012. Src-dependent autophagic degradation of Ret in FAK-signalling-defective cancer cells. EMBO Rep. 13, 733–740.
Shi, C.S., Shenderov, K., Huang, N.N., et al., 2012. Activation of autophagy by inflammatory signals limits IL-1 beta production by targeting ubiquitinated inflammasomes for destruction. Nat. Immunol. 13, 255–263.
Spencer, S.L., Gaudet, S., Albeck, J.G., et al., 2009. Non-genetic origins of cell-to-cell variability in TRAIL-induced apoptosis. Nature 459, 428–432.
Tresse, E., Giusti, C., Kosta, A., et al., 2008. Autophagy and autophagic cell death in *Dictyostelium*. Methods Enzymol. 451, 343–358.
Wang, W.J., Wang, Y., Chen, H.Z., et al., 2014. Orphan nuclear receptor TR3 acts in autophagic cell death via mitochondrial signaling pathway. Nat. Chem. Biol. 10, 133–140.
Wirawan, E., Vande Walle, L., Kersse, K., et al., 2010. Caspase-mediated cleavage of Beclin-1 inactivates Beclin-1-induced autophagy and enhances apoptosis by promoting the release of proapoptotic factors from mitochondria. Cell Death Dis. 1, e18.
Xu, Y., Yuan, J., Lipinski, M.M., 2013. Live imaging and single-cell analysis reveal differential dynamics of autophagy and apoptosis. Autophagy 9, 1418–1430.
Young, M.M., Takahashi, Y., Khan, O., et al., 2012. Autophagosomal membrane serves as platform for intracellular death-inducing signaling complex (iDISC)-mediated caspase-8 activation and apoptosis. J. Biol. Chem. 287, 12455–12468.
Yousefi, S., Perozzo, R., Schmid, I., et al., 2006. Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. Nat. Cell Biol. 8, 1124–1132.
Yu, L., Wan, F., Dutta, S., et al., 2006. Autophagic programmed cell death by selective catalase degradation. Proc. Natl. Acad. Sci. U.S.A. 103, 4952–4957.
Zhaorigetu, S., Wan, G., Kaini, R., et al., 2008. ApoL1, a BH3-only lipid binding protein, induces autophagic cell death. Autophagy 4, 1079–1082.

CHAPTER

8

The Role of Autophagy and Mitophagy in Mitochondrial Diseases

Marina Villanueva Paz, David Cotán, Mario D. Cordero, Juan Garrido Maraver, Manuel Oropesa-Ávila, Mario de la Mata, Ana Delgado Pavón, Elisabet Alcócer Gómez, Isabel de Lavera and José A. Sánchez Alcázar

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Abstract

Mitochondrial diseases are a group of rare genetic disorders with a very heterogeneous origin caused by total or partial dysfunction of mitochondria, the organelles that produce most of the energy of the cell. ATP shortage and excess production of ROS are the main pathogenic factors that cause most of the clinical manifestations of mitochondrial diseases. Recent reports have demonstrated that these pathological signals promote the induction of bulk autophagy and/or mitophagy in different models of mitochondrial disease.

The term "autophagy" is used to describe lysosomal-mediated degradation of intracellular contents including damaged or excessive organelles through the formation of a double-membrane structure known as the autophagosome. In contrast, mitophagy refers to the selective degradation of mitochondria by autophagy and normally needs complementary factors as low mitochondrial membrane potential and Parkin translocation to mitochondria. Most of the investigators hypothesize that both bulk autophagy and mitophagy have a protective role in mitochondrial disease, since the accumulation of damaged mitochondria and other toxic aggregates causes a worsening of cell pathophysiology. Indeed, mitophagy could modulate the percentage of heteroplasmy of the mitochondrial DNA (mtDNA) mutations, which is directly related with the pathophysiology of the disease. However, regulation of autophagy and mitophagy in the cell appears to be essential for the balance between survival and cell death, since autophagy is a catabolic process that could potentially be used by the cell to its self-destruction, as well as massive mitophagy can suppose an extensive loss of mitochondrial mass resulting in the bioenergetics collapse of the cell.

INTRODUCTION

Mitochondria are vital organelles for every nucleated cell as they generate ATP by the oxidative phosphorylation (OXPHOS) system which is necessary for the energetic survival of the cell. They are also important for several aspects of the metabolism of the cell, since they regulate apoptosis, calcium homeostasis, and the response against oxidative stress mainly caused by the mitochondrial production of reactive oxygen species (ROS). Due to the important functions of mitochondria in the cell, mitochondrial dysfunctions cause a great variety of diseases which can affect almost all the tissues in the body. Furthermore, there is a huge variety in the symptoms and severity of mitochondrial diseases from life-threatening to almost asymptomatic, sometimes taking both extremes in members of the same family. Although the clinico-pathological and molecular features of mitochondrial diseases have been extensively studied in recent years, many physiopathological aspects need further research. In addition, there are no effective treatments for mitochondrial disease, although current therapies may alleviate specific clinical symptoms and prevent partially disease progression (Parikh et al., 2009).

Autophagy and mitophagy are currently gaining importance since the promotion of these processes could be protective for the progression of some mitochondrial diseases.

Autophagy is a catabolic process that results in the autophagosomic-lysosomal degradation of bulk cytoplasmic contents including cell organelles (Mizushima, 2007). In contrast, mitophagy regulates specifically the number of functional mitochondria in the cell performing a quality control that would eliminate damaged or defective mitochondria (Lemasters, 2005). Autophagy functions as a stress adaptation mechanism which generally promotes cell survival by preventing cell death by apoptosis (Goldman et al., 2010). In the same manner, mitophagy exerts a protective role on cells in many situations because it selectively degrades damaged mitochondria with high levels of ROS and low membrane potential that

impair cellular homeostasis. However, activation of mitophagy must be accompanied by an increase of mitochondrial biogenesis as a compensatory mechanism to prevent massive mitochondrial loss (Kim et al., 2007).

MITOCHONDRIAL DISEASES

Mitochondrial diseases are a group of rare genetic disorders of very heterogeneous origin. The estimated prevalence of these diseases is at least 1 in 5000 people, even though it could be much higher (Schaefer et al., 2004). In general, the term "mitochondrial disease" refers to those disorders caused by total or partial dysfunction of the mitochondrial electron transport chain coupled to the oxidative phosphorylation (OXPHOS) system. This alteration leads to an energy deficiency in the cell (Hofmann and Bauer, 2006). Genes responsible for mitochondrial metabolism (including OXPHOS components) are encoded by both nuclear and mitochondrial DNA (mtDNA); therefore, mitochondrial diseases can be caused by mutations in both genomes. This double genetic control of mitochondrial function gives rise to different patterns of inheritance of mitochondrial diseases and, consequently complicates their characterization and diagnosis. Furthermore, mitochondrial diseases have a great variety of phenotypes that are mainly expressed in organs with higher energy demand such as brain, muscle, heart, kidney, and endocrine system (Wallace, 1999). Additionally, it is still difficult to establish the relationship between the molecular pathology of mitochondrial diseases and the variety of phenotypes associated with them (Sproule and Kaufmann, 2008).

Sequencing the human mitochondrial genome in 1981 allowed the further identification of numerous pathogenic point mutations, representing a breakthrough in the molecular diagnosis and understanding of multiple mitochondrial diseases (Anderson et al., 1981). Two types of mutations of mtDNA can be distinguished. First, point mutations that can affect 1 of the 13 genes encoding mtDNA proteins, the 2 genes encoding ribosomal RNA (rRNA) or the 20 genes encoding transfer RNA (tRNA) which globally affect mitochondrial protein synthesis. For example, MELAS and MERRF syndromes mainly caused by point mutations in genes codifying tRNAs (Goto et al., 1990; Shoffner et al., 1990) or NARP and LHON syndromes caused by mutations in genes encoding the mtDNA proteins ATP6 and ND1, respectively (Holt et al., 1990; Howell et al., 1991). Second, some mitochondrial diseases are caused by large deletions, insertions, or mtDNA duplications (Hofmann and Bauer, 2006). For example, Kearns–Sayre and Pearson syndromes are mitochondrial diseases caused by a single, large-scale deletion which includes several genes (Rötig et al., 1990; Zeviani et al., 1988). Patients harboring pathogenic mtDNA mutations often have a mixture of wild-type and mutated molecules, a condition called heteroplasmy. The mutation load can vary widely between members of the same family and in different cells of particular tissues. This uneven distribution of mutated mtDNA leads to a mosaic respiratory chain deficiency in affected tissues that is an important determinant in disease pathophysiology (Park and Larsson, 2011). Thus, clinical features of mitochondrial diseases are very complex because of the wide range of tissues affected and the variety of phenotypes involved, depending of the mutational load, patient genetic background, and the existence of other mutations. Most common features in mitochondrial diseases involve several clinical

areas: neurological symptoms (migraine, epilepsy, myopathy, dementia, ataxia, etc.), cardiomyopathy, dysphagia, respiratory problems (hypoventilation, apnea, pneumonia), diabetes, ophtalmoplegia and optic atrophy, muscular problems (ptosis, dystonia, myopathy), and less common renal tubular defects and hepatic failures (Taylor and Turnbull, 2005).

Molecular Pathophysiology of Mitochondrial Diseases

Due to the heterogeneous origin of mitochondrial diseases, the understanding of the pathogenic mechanisms of mitochondrial diseases is still very limited. ATP shortage and ROS excess are the main pathogenic factors that cause most of clinical manifestations of mitochondrial diseases (DiMauro and Hirano, 2009; Shepherd et al., 2006). In most of the cases, ATP shortage is due to a dysfunction in the OXPHOS system. Due to the reduction of ATP synthesis capacity, cells with mitochondrial dysfunctions show a chronic stage of energy insufficiency which finally induces a disruption of cell homeostasis.

Other specific molecular features of mitochondrial diseases are:

i. Decrease in the activity of the complexes of the OXPHOS system.
ii. Increase in glycolytic pathways, anaerobic metabolism of glucose.
iii. Increase in lactate production.
iv. Lower respiratory capacity per mitochondria.
v. Lower mitochondrial membrane potential ($\Delta\Psi$).
vi. Increase in cytosolic calcium levels.
vii. Increase in ROS production.
viii. Premature aging.
ix. Decrease in coenzyme Q_{10} levels.
x. Reduction of insulin secretion.
xi. Increase of apoptosis levels.

Besides these characteristics, it has been demonstrated that several cellular models of mitochondrial diseases show high levels of bulk autophagy and/or mitophagy. These process are gaining importance nowadays due to that their regulation could be a promising therapeutic target for mitochondrial dysfunction. There is not a common agreement about the presence of autophagy and/or mitophagy in mitochondrial diseases and different authors show diverse hypothesis about the importance and role of both processes.

AUTOPHAGY

Autophagy is an intracellular degradation system by which cytosol and organelles are sequestered within double-membrane vesicles (pre-autophagosomes) that deliver the contents to the lysosome/vacuole for hydrolytic degradation and recycling of the resulting macromolecules. Autophagy is typically activated by fasting and nutrient deprivation for generating amino acids and metabolic intermediates to maintain ATP production (Levine and Kroemer, 2008). Furthermore, autophagy allows the removal of defective or useless organelles. In that context, some authors describe bulk autophagy as a survival mechanism of the cell (Goldman et al., 2010). Therefore, an appropriate regulation of autophagy

is thus essential for cellular homeostasis, since only aberrant or defective organelles have to be degraded. The molecular mechanism underlying autophagy has been extensively researched in the past decade, and the genes participating in this process, denoted ATGs, were found to be conserved from yeast to humans (Klionsky et al., 2003; Tsukada and Ohsumi, 1993). There is a controversy about considering if autophagy promotes or prevents cell death. If autophagy removes damaged organelles that would otherwise activate caspases and apoptosis, then autophagy could be protective. On the other hand, some authors describe autophagy as other way of programmed cell death different of apoptosis, since autophagy is an essentially catabolic process that could potentially be used by the cell to its self-destruction (Debnath et al., 2005). Therefore, regulation of autophagy in the cell appears to be essential for the balance between survival and cell death.

Regulation of Autophagy in Mitochondrial Diseases

Different human and murine models of mitochondrial diseases showed an increased autophagy compared with control situations. Autophagy could be induced as a compensatory mechanism to obtain energy or to eliminate damaged organelles whose accumulation could exacerbate the mitochondrial dysfunction. In a mouse model of mitochondrial myopathy due to mutation in the mitochondrial helicase Twinkle, evidence of an increased recycling of mitochondria through autophagy in muscle fibers has been observed (Tyynismaa et al., 2005). Furthermore, an increase of autophagy has also been observed in muscle biopsies of patients harboring m.8344 A> G mutation, the most common mutation that causes MERRF syndrome (Yuan et al., 2013). Recently, the role of bulk autophagy in fibroblast models of mitochondrial dysfunction due to deficiencies in OXPHOS system has been described (Morán et al., 2014). The authors found an accumulation of autophagosomes and lysosomes in the cells, but a selective process of mitochondria degradation could not be detected. The increase of autophagic markers in these models of mitochondrial disease can be due to an impairment of the autophagic flux, which causes an accumulation of autophagosomes. This block of lysosome flux can be caused by a depletion of ATP levels, a characteristic of mitochondrial dysfunction, because ATP is necessary to maintain lysosome function and to seclude the cytosolic components (Cotán et al., 2011; Morán et al., 2014).

Protective Role of Autophagy in Mitochondrial Diseases

The protective role of autophagy has been confirmed in multiple cellular types and situations by a large number of studies. Among others, it has been observed that autophagy activation has a neuroprotective role during brain damage (Jing et al., 2012). In fact, protection of autophagy has been suggested to be a possible future therapy for brain disorders like schizophrenia (Merenlender-Wagner et al., 2014). However, currently there is not an agreement about if autophagy has a protective or damaging role in mitochondrial diseases. Recently, the protective role of autophagy in diseases with mitochondrial dysfunctions such as osteoarthritis has been suggested. Autophagy protects chondrocytes with mitochondrial dysfunctions from oxidative stress, therefore regulation of autophagy could be a promising therapeutic target for aging-related skeletal disorders (López de Figueroa et al., 2015). In the same way, deficient or disrupted autophagy is characteristic of other diseases that

present mitochondrial dysfunction, mostly neurodegenerative disorders, since deficient elimination of toxic aggregates in neurons and other cell types promotes cellular stress and death. Dysregulation of autophagy is a contributing factor of chronic, disable diseases such as Alzheimer's disease (AD), Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (Ghavami et al., 2014).

MITOPHAGY

The term "mitophagy" was coined by J. J. Lemasters in 2005 to define the selective degradation of mitochondria by autophagy (Lemasters, 2005). Damaged or useless mitochondria are selectively degraded by a regulated and complex process which allows regulating the number of functional mitochondria in the cell, both for satisfying the metabolic demand and performing a mitochondrial quality control. Within the mitochondria the primary ROS produced is superoxide ($O_2^{\bullet -}$), most of which is converted to hydrogen peroxide (H_2O_2) by the action of superoxide dismutase. The production of superoxide by mitochondria has been localized to several enzymes of the electron transport chain including Complexes I and III and glycerol-3-phosphate dehydrogenase (Lambert and Brand, 2009). If the mitochondria is defective or cellular respiration is inhibited, the level of ROS produced by mitochondria increases. Due to the production of these reactive species, mitochondria are highly exposed to oxidative stress which causes the opening of permeability transition pores in the inner membrane and the permeability of mitochondrial membranes to molecules of less than 1.5 KDa. Induction of the mitochondrial permeability transition (MPT) can lead to mitochondrial swelling and cell death through apoptosis or necrosis depending on the particular biological situation (Lemasters et al., 2009). Moreover, induction of MPT causes mitochondria to become uncoupled and depolarized, and as a consequence, mitochondria show a defective synthesis of ATP. In addition, ROS are implicated in genotoxicity and cause the accumulation of mutations in the mtDNA. The accumulation of oxidative damage in the mitochondria over time causes the dysfunction of the organelle and can even cause cell apoptosis due to release of proapoptotic proteins. Therefore, mitochondrial quality control must occur in the cell to eliminate ancient or damaged mitochondria that produces high levels of ROS. This process is mainly performed by mitophagy (Kim et al., 2007). In fact, ROS-induced mitochondrial damage may be an important upstream activator of mitophagy (Wang et al., 2012).

Understanding of mitophagy mechanisms at the molecular level was pioneered in yeast (Kanki and Klionsky, 2008, 2010; Kanki et al., 2009; Okamoto et al., 2009). These studies identified genes that are specifically required for mitophagy, but not for other forms of autophagy. Atg32 is of particular interest because of its role as a mitophagy receptor. It is anchored to the outer membrane of mitochondria and recruits of Atg8, a protein essential for autophagosome formation. Mitochondria-derived ROS, at low concentrations, may act as signaling molecules and trigger mitophagy through regulation of Atg4, an essential cysteine protease in the autophagic pathway (Scherz-Shouval et al., 2007). The formation of autophagosomes depends on Atg4-mediated cleavage of Atg8 and its subsequent conjugation to phosphatidyl ethanolamine at autophagosomal membranes.

In mammals, three different mechanisms of mitophagy are known (Youle and Narendra, 2011). First of all, most mammals' erythrocytes lose their mitochondria during maturation by mitophagy. The NIX protein, located in the outer mitochondrial membrane, has a WXXL-like domain in its cytosolic side, which is capable of binding to the LC3 protein, a specific autophagosome membrane marker. The binding of NIX and LC3 promotes the isolation of mitochondria in the phagophore (Novak et al., 2010; Sandoval et al., 2008).

The second mitophagic mechanism in mammals is considered to be induced by depolarization of the mitochondrial membrane due to damage accumulation and the action of harmful agents in mitochondria, such as ROS (Wang et al., 2012). This process is dependent on the interaction between PINK1 kinase and ubiquitin ligase Parkin. In 2008, Parkin recruitment to depolarized mitochondria and the subsequent degradation of mitochondria by mitophagy was described (Narendra et al., 2008). Parkin is a cytosolic E3 ubiquitin ligase with an ubiquitin-like domain in its N-terminal end, which allows the transference of ubiquitin to lysine residues of target proteins, including Parkin itself (Springer and Kahle, 2011). Parkin catalyzes a range of different ubiquitination events, from monoubiquitination (which has various cellular functions) until polyubiquitination of proteins, which results in proteosome delivery for protein degradation. PINK1 is a serine threonine kinase whose cellular localization is not yet clear, as it is found both in the cytosol and some mitochondrial compartments, most likely anchored to the outer mitochondrial membrane by a transmembrane domain. Under steady-state conditions, PINK1 and Parkin regulate cell morphology by interacting with mitochondrial fusion/fission machinery. In these conditions, PINK1 is imported to mitochondria in a voltage-dependent manner and is rapidly degraded in the intermembrane space by proteolytic processing in a mitochondrial membrane potential-dependent manner. Thus, while PINK1 is normally kept at low levels on healthy mitochondria, it rapidly and selectively accumulates on mitochondria that have lost membrane potential (Matsuda et al., 2010; Narendra and Youle, 2011). Under dissipation of mitochondrial membrane potential conditions due to mitochondrial damage (ROS), loss of cellular respiration, mtDNA mutations, use of uncoupling agents, etc., PINK1 is recruited to the outer mitochondrial membrane by the TIM/TOM complex and it is stabilized in a voltage-dependent manner. As a consequence, PINK1 is kept in the outer membrane of depolarized mitochondria (Matsuda et al., 2010). In these conditions, PINK1 recruits Parkin to mitochondria through direct phosphorylation, increasing Parkin ubiquitin ligase capacity. Once Parkin is phosphorylated, it remains in the outer mitochondrial membrane where different mitochondrial proteins like VDAC1 and mitofusins 1 and 2 will be ubiquitinated (Gegg et al., 2010; Geisler et al., 2010). Ubiquitination of mitochondrial proteins promotes the recruitment of adaptor proteins such as p62/SQSTM1. This protein is able to bind ubiquitinated proteins of the outer membrane of mitochondria (through its UBA domain, associated with ubiquitin) and autophagic proteins such as LC3 (through its interaction domain LIR). The interaction between p62 and LC3 allows binding of depolarized mitochondria with the phagophore (Geisler et al., 2010). The adaptor protein HDAC6 has also an important role in mitophagy. This cytosolic histone deacetylase is able to bind ubiquitinated proteins to the microtubule transport system (by interaction with dynein protein) and facilitates the concentration and aggregation of depolarized mitochondria near the nucleus. Finally, in this particular area, this structure called "mito-agresomme"

is engulfed by the forming autophagosomes that later fuse with lysosomes (Springer and Kahle, 2011).

Finally, as a third mechanism of mitophagy, the existence of a Parkin-independent mitophagic mechanism mediated by iron metabolism has been recently suggested (Allen et al., 2013). It has been observed in different cell lines that cellular iron chelation induces mitophagy without altering the normal conditions of PINK1 and Parkin. However, more studies are needed to determine the influence of iron on mitophagy.

Mitophagy and Mitochondrial Dynamics Regulate Mitochondrial Quality Control

Currently, how dysfunctional mitochondria are distinguished from functional ones at the molecular level is yet not fully understood. However, it is known that mitochondria have different ways to perform a quality control. First of all, as dynamic organelles, they are constantly undergoing fission and fusion to adapt toward changes in the cellular environment. Fission produces small spherical mitochondria, whereas fusion produces tubular or elongated mitochondria. It is well established that mitochondrial dynamics play a crucial role in the quality control of mitochondria (Youle and van der Bliek, 2012). When mitochondria are functional, fusion and fission occur in a constant and balanced manner to adapt the morphology of the mitochondrial network to the metabolic needs of the cell (Suen et al., 2008). Healthy mitochondria form elongated tubules that continually divide and fuse to form a dynamic interconnecting network. The molecular machinery that mediates this organelle fission and fusion is necessary to maintain mitochondrial integrity, perhaps by facilitating DNA or protein quality control (Amchenkova et al., 1988). The fusion process is critical for maintenance of mitochondrial function, as interruption of mitochondrial fusion results in a loss of inner mitochondrial membrane potential (Chen et al., 2003). In fact, mitochondrial fusion allows the spreading of metabolites and mitochondrial gene products throughout the entire mitochondrial compartment. This serves to optimize mitochondrial function and counteracts the accumulation of mitochondrial mutations during aging. Therefore, it is suggested that mitochondrial fusion prevents the induction of mitophagy (Twig and Shirihai, 2011).

Mitochondrial fusion in mammals is mediated by the proteins mitofusin 1 and mitofusin 2 (Mfn1 and Mfn2) and optic atrophy 1 (OPA1). Mfn1 and Mfn2 are dynamin-related GTPases that are responsible for fusion of outer mitochondrial membranes. OPA1 is also a dynamin-related GTPase, which is responsible for fusion of inner mitochondrial membranes (Ni et al., 2014). In fact, mutations in Mnf2 can cause neurodegenerative diseases such as Charcot–Marie–Tooth neuropathy type 2A (Züchner et al., 2004), suggesting that it is necessary to preserve the correct function of mitochondrial fusion to satisfy the energy demands of the cell. In contrast, mitochondrial fission plays an important role in the removal of damaged organelles by selective autophagy. As soon as individual parts of the mitochondrial network become dysfunctional, fission events induced by proteins such as Fis1 and DRP1, permit damaged mitochondria to become spatially isolated. Thus, dysfunctional mitochondria are distinguished on a morphological basis from the rest and can be presumably degraded by selective autophagy (Gomes and Scorrano, 2008). Since mitochondrial fission may be necessary for the onset of mitophagy, ubiquitination of Mfn2 by Parkin could be

important for dysfunctional mitochondria signaling (Twig et al., 2008). In fact, it has been hypothesized that Parkin facilitates mitophagy by eliminating functional mitofusin and increasing the fission process. Thus, there is an increase in the number of small and isolated mitochondria which can be easily handled by the phagosome. However, mitochondrial fission is necessary but not sufficient for mitophagy activation since mitochondria have to be depolarized and/or dysfunctional to prevent mitochondrial fusion and induce mitophagy (Ding and Yin, 2012).

Besides mitochondrial fusion and fission, mitochondrial mobility through the cytoskeleton is very important for mitochondrial removal and quality control. Mitochondria make use of the kinesin-dinein motor to move along the microtubules. The attachment between the mitochondria and microtubules is performed by the interaction between a mitochondrial membrane protein (Miro) and an adapter protein (Milto) (Saxton and Hollenbeck, 2012). Although it is not clear if mitochondrial motility prevents mitophagy, it has been demonstrated in HeLa cells that knockdown of Miro accelerate the mitochondrial removal by mitophagy (Liu et al., 2012). Moreover, Parkin ubiquitinates Miro, preventing mitochondrial movement and facilitating mitochondrial isolation (Wang et al., 2011). Other less known mechanisms to perform mitochondrial quality control have been suggested such as Parkin-independent mitophagic mechanisms or mitochondrial spheroids formation (Ni et al., 2014). However, it is needed further investigation to understand the importance of these mechanisms in mitochondrial turnover.

Mitophagy and Mitochondrial Biogenesis

Massive mitophagy of dysfunctional mitochondria can suppose a loss of mitochondrial mass resulting in the energy disruption of the cell. The lack of energy input would increase mitochondrial damage and eventually it would trigger apoptosis. Therefore, it is reasonable to hypothesize that mitophagy in the presence of defective mitochondrial function must be accompanied by an increase in mitochondrial biogenesis to preserve cell homeostasis. If mitochondrial elimination is not compensated by mitochondrial biogenesis, mitophagy is no longer protective and becomes detrimental for cell survival. For example, during subacute sepsis in the liver, mitochondrial function is impaired and mitochondria are removed by autophagy. However, clearance of mitochondria is followed by mitochondrial replenishment (Crouser et al., 2006). Moreover, it has been observed an increase in mitochondrial biogenesis after promoting mitochondrial dysfunction by oxidant injury in renal cells (Rasbach and Schnellmann, 2007).

Mitochondrial biogenesis can be triggered by characteristic signals of mitochondrial dysfunction. When cells show low levels of ATP (due to mitochondrial dysfunction, for example), the AMP/ATP ratio increases and AMPK is activated, which then promotes the activation of several transcription factors implicated in mitochondrial biogenesis such as PGC1-α (Hardie et al., 2012). Therefore, although mitochondrial biogenesis and mitophagy are opposite processes, they must be highly linked and coordinated to determine mitochondrial function, structure and homeostasis. In fact, both processes regulate cellular adaptations to mitochondrial dysfunction. Furthermore, under lack of nutrients or cellular stress conditions, activated AMPK phosphorylates the autophagic protein UKL-1 and also promotes autophagy. Thus, AMPK or UKL-1 loss in mammals causes defects in mitophagy

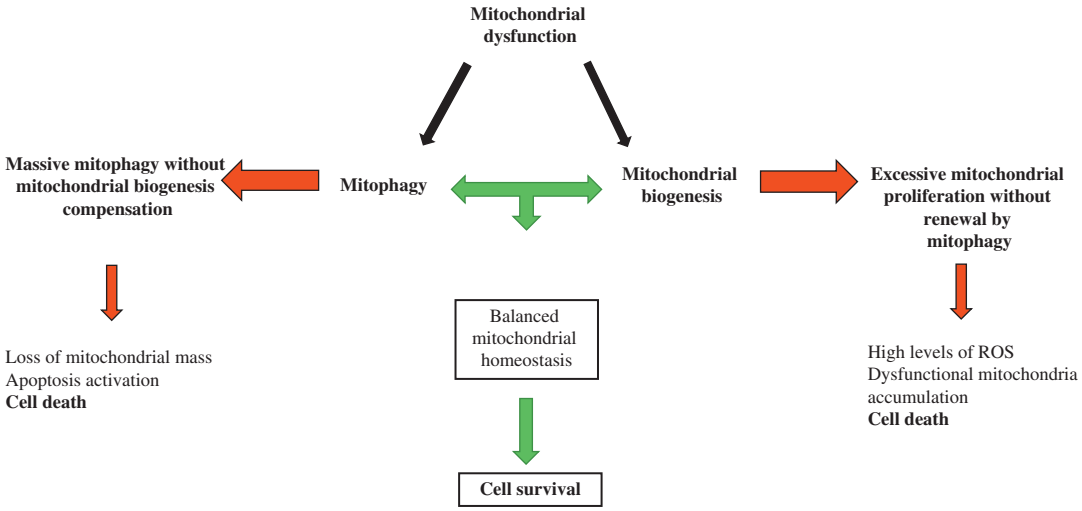

FIGURE 8.1 Scheme, showing the relationship among mitophagy, mitochondrial biogenesis, and cell death.

under stressful situations. Interestingly, AMPK is capable of initiating a dual response that promotes mitophagy as well as mitochondrial biogenesis (Palikaras and Tavernarakis, 2014). It is important to highlight that mitochondrial biogenesis in the absence of balanced mitophagy to remove defective mitochondria may be maladaptive due to increased ROS generation, accumulation of damaged mitochondria, and finally cell death. Therefore, a balanced function of these two processes is essential for cellular adaptation and dysfunction resistance (Figure 8.1).

MITOPHAGY IN MITOCHONDRIAL DISEASES

In the same way that autophagy, mitophagy seems to exert a protective role on cell homeostasis in many situations. Thus, it has been demonstrated that mitochondrial autophagy is activated during sepsis and the activity of Parkin is implicated in cardiac recovery (Piquereau et al., 2013). In addition, removal of aberrant mitochondria has been shown to play a protective role in age-related neurodegenerative disorders such as Parkinson disease. Since Parkin is essential for mitophagy, Parkinson's disease may be at least in part associated with impaired elimination of dysfunctional mitochondria (Vives-Bauza and Przedborski, 2011). Furthermore, in other neurodegenerative diseases such as AD, an alteration in mitochondrial function has been suggested. An imbalance in oxidative status, bioenergetics deficit, decrease in cytochrome oxidase (COX), and an increase in oxidative damage occur prior to senile plaque formation (Maurer et al., 2000; Nunomura et al., 2001; Yao et al., 2009). Currently, the role of fission/fusion and mitophagy in AD is gaining importance. Although there is evidence of mitophagy in AD (Moreira et al., 2007), it is not clear if a correct degradation of sequestered mitochondria is being performed or if all the degraded

mitochondria are damaged. In addition, it has been suggested that mitochondrial fission is upregulated in AD, probably in an attempt to segregate damaged mitochondria for degradation by mitophagy. However, if mitophagy is efficient or not has to be clarified (Santos et al., 2010).

A specific characteristic of cells from patients with primary mitochondrial disorders is a decrease in the mitochondrial membrane potential due to deficiencies in the electron flow through the respiratory chain. This fact suggests that depolarized mitochondria could be selectively eliminated by mitophagy in these patients. Thus, it has been observed an augmented mitophagic response in MELAS and MERRF fibroblasts. In both cases, cells show low membrane potential, high level of ROS, and CoQ deficiency. In fact, CoQ supplementation restored mitochondrial morphology, membrane potential, and ROS levels, as well as autophagosome/autophagolysosome accumulation (Cotán et al., 2011; Mata et al., 2012; Rodríguez-Hernández et al., 2009).

Protective Role of Mitophagy in Mitochondrial Diseases

The protective role of mitophagy in physiological and physiopathological situations has been described by many authors in several models. PINK1 and Parkin are essential for the quality control of mitochondria and the loss or dysfunction of anyone of them causes severe impairments in mitochondria function. Experiments with PINK1$^{-/-}$ mice showed impaired mitochondrial respiration and an increase in large mitochondria which can be related with the function of PINK1 in promoting fission and mitophagy (Gautier et al., 2008). These effects were exacerbated with mice age suggesting that mitophagy has an important role in aging. In addition, it has been demonstrated that alterations in mitophagy due to PINK1 silencing diminish cell resistance under hyperoxia situations and oxidative stress in the lung endothelium (Zhang et al., 2014).

Mitophagy is a process closely related to the expression levels of Parkin in the cell. Some authors defend the hypothesis that cells with mutated mitochondrial DNA have decreased levels of PINK1 and Parkin, possibly due to transcriptional inhibition mechanisms, although they are not yet known. In fact, it has been observed that cybrids cells with mitochondrial DNA mutation (A3243G) have less Parkin expression levels than wild-type cybrids cells which impairs the elimination of dysfunctional mitochondria (Gilkerson et al., 2012).

Mitophagy stimulation could have a large potential as a therapeutic strategy in mitochondrial diseases or diseases with mitochondrial dysfunctions. For example, some authors have proposed that the absence of selective mitophagy is a cause of the pathophysiology of Parkinson's disease. According to these authors, the low endogenous level of Parkin due to its self-ubiquitination, among other process, prevents mitophagy, and as a consequence dysfunctional mitochondria cannot be renewed. This accumulation of damaged mitochondria causes a worsening of cell pathophysiology (Rakovic et al., 2013). In order to observe mitophagy in cell cultures various situations must be given: presence of low mitochondrial membrane potential and/or high levels of ROS, activation of the autophagic machinery, and sufficient levels of Parkin/PINK1. Furthermore, some authors also suggest that the inhibition of the mammalian target of rapamycin complex I (mTORC1) is essential for mitophagy activation (Gilkerson et al., 2012).

It has been showed that promoting mitophagy ameliorates mitochondrial dysfunction in diverse physiological and physiopathological situations. For example, mitophagy is considered to be an adaptive metabolism to prevent oxidative damage and hypoxia (Zhang et al., 2008). It has been observed after cerebral ischemia that treatment with an autophagy activator, rapamycin, promotes a reduced infarct volume, improved neurological outcomes and inhibited mitochondrial dysfunction in experimental animals. The beneficial effects of rapamycin were due to an increase in mitophagy (Li et al., 2014). In addition, promotion of mitophagy by rapamycin has demonstrated to be protective against the effects of rotenone, a complex I inhibitor that causes mitochondrial dysfunction (Pan et al., 2009). Moreover, it has been demonstrated that disruption of mitophagy in cellular models of MELAS or MERRF disease results in cell death by apoptosis, suggesting a protective role of mitophagy in MELAS and MERRF fibroblasts (Cotán et al., 2011; Mata et al., 2012). Furthermore, prevention of mitophagy in CoQ deficient cells by chemical compound such as 3-methyl adenine or wortmannin or by genetic knockdown of ATG genes caused apoptotic cell death (Rodríguez-Hernández et al., 2009). In addition, it has been demonstrated that the induction of CoQ deficiency in $Atg5^{-/-}$ mouse embryonic fibroblasts induced apoptosis, suggesting again the protective role of mitophagy.

Mitophagy and Regulation of Heteroplasmy in Mitochondrial Diseases

Each human cell contains thousands of copies of mtDNA that are usually identical (homoplasmy). However, patients with mitochondrial diseases caused by mutations of mtDNA frequently present a mixture of mutant and wild-type mtDNA within each cell. In general, it is accepted that high heteroplasmy is associated with severe clinical presentations (Wallace and Chalkia, 2013). Mutational load of tissues varies, usually being higher in muscle (the best choice for diagnosis) and buccal mucosa and lower in blood cells. The mutational load can be also detected in urine sediment and dermal fibroblasts (Finsterer, 2007). Mutational load increases with age in most tissues. However, in lymphocytes mutational load decreases with age, due to a selection of positive clones that have more wild-type mtDNA. This fact explains why there is no correlation between the degree of heteroplasmy detected in blood and clinical symptoms of the patient (Chinnery et al., 1997). An important role of mitophagy in mitochondrial diseases is its capability to modulate the percentage of heteroplasmy. Since the percentage of heteroplasmy is directly proportional to the severity of the mitochondrial disease, modulating heteroplasmy could be a promising therapeutic strategy. Different methods have been proposed to modulate and/or decrease the percentage of heteroplasmy in diverse models of mitochondrial diseases. The use of mitochondrial-targeted restriction endonucleases allows decreasing the percentage of heteroplasmy *in vivo* in mice models (Bacman et al., 2012; Bayona-Bafaluy et al., 2005). However, due to the problems and possible side effects of gene therapy and the use of viral vectors, other strategies to diminish heteroplasmy are required. Mitophagy specifically eliminates damaged mitochondria, with high level of ROS and/or low membrane potential. Presumably, in mitochondrial diseases, mitochondria with these characteristics have higher percentage of heteroplasmy. Therefore, stimulating mitophagy seems to be a possible strategy to decrease the level of heteroplasmy, since only defective mitochondria with high percentage of heteroplasmy will be eliminated and healthy mitochondria will proliferate. Thus, mitophagy may act as a segregation mechanism of heteroplasmic mutations.

Different strategies have been performed to stimulate mitophagy in diverse models of mitochondrial diseases. For example, induction of mitophagy by rapamycin treatment led to the reduction of the mutational load of a cybrid cell culture harboring the heteroplasmic mutation m.11778G>A, the most common mutation which causes LHON (Dai et al., 2014). In the same way, overexpression of Parkin in cybrids with 80% of mutated mtDNA (COXI, cytochrome c oxidase I mutation) led to an increase in mitophagy which allows a reduction of the mutational load up to 26.7% and, most important, this reduction remained over time (Suen et al., 2010). Moreover, cytochrome c oxidase activity was restored in cybrid cells enriched for wild-type mtDNA indicating that decrease of heteroplasmy could improve the physiopathology of the disease. Therefore, upregulation of Parkin expression may be beneficial for hereditary mitochondrial diseases. These findings also indicate that endogenous Parkin levels may be limiting for the negative selection of dysfunctional mitochondria in some cell types, and thus, low levels of Parkin in these cells may impair the physiological elimination of damaged mitochondria.

Several authors have hypothesized that mitochondrial dysfunction *per se* is not sufficient to recruit mitophagic factors to damaged mitochondria and induce mitophagy. Although high levels of ROS and/or low mitochondrial membrane potential are necessary to promote mitophagy, Parkin expression levels may be also critical to allow the degradation of dysfunctional mitochondria. In fact, it has been shown that cybrids A3242G with low expression levels of Parkin do not show an increase in autophagy and/or mitophagy when they are treated with rapamycin (Gilkerson et al., 2012). Moreover, mitophagy is also closely related with mitochondrial dynamics (fusion and fission). It has been suggested that mitochondrial fission (which promotes mitophagy) prevents the increase of heteroplasmy in cell models of mitochondrial diseases. In fact, studies with cell cultures of rhabdomyosarcoma harboring a 80% heteroplasmy of m.3243A> G have shown that the knockdown of DRP-1 (starting protein of mitochondrial fission) causes an increase of percentage of heteroplasmy from 80% to 96% (Malena et al., 2009).

CONCLUSIONS

Under steady-state conditions, cells utilize autophagy to eliminate dysfunctional and superfluous cytoplasmic components to maintain cellular homeostasis. In particular, mitophagy selectively eliminates damaged mitochondria with high level of ROS and/or low membrane potential. In a mitochondrial dysfunction background, several authors have described the presence of increase autophagy and/or mitophagy. Since mitophagy needs the activation of the autophagy machinery, biological models of mitochondrial diseases showing mitophagy activation have also increased common autophagic markers. However, some cellular models of mitochondrial dysfunction have been reported to show increased bulk autophagy without the presence of mitophagy. These divergent results can be explained by the different molecular characteristics of the diverse mitochondrial diseases cell models. For instance, mitophagy induction needs decreased $\Delta\Psi$, increased ROS levels and mitochondrial isolation (fission), in addition of induction of the autophagy machinery.

Although there is not yet agreement, it seems that autophagy and mitophagy have a protective role in mitochondrial diseases, since disruption of these processes can promote an

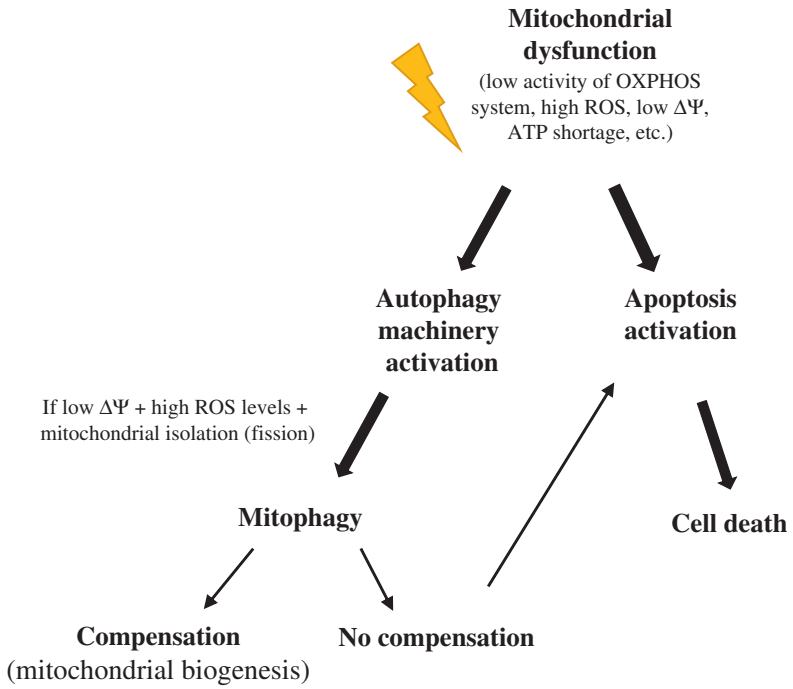

FIGURE 8.2 Scheme showing the relationship among mitochondrial dysfunction, autophagy and mitophagy, and cell death.

increase in the severity of the pathophysiological alterations or even cell death. Moreover, induction of mitophagy results in a decrease of the percentage of heteroplasmy of some mtDNA mutations and significant improvement of mitochondrial function. However, in cells with defective mitochondrial function, mitophagy must be accompanied by an increase in mitochondrial biogenesis to preserve cell homeostasis (Figure 8.2).

Alongside gaining an understanding in the molecular mechanisms and the implications of both autophagy and mitophagy in mitochondrial dysfunction, future research should focus on how to use them as therapeutic targets in mitochondrial diseases.

References

Allen, G.F.G., Toth, R., James, J., Ganley, I.G., 2013. Loss of iron triggers PINK1/Parkin-independent mitophagy. EMBO Rep. 14, 1127–1135.

Amchenkova, A.A., Bakeeva, L.E., Chentsov, Y.S., et al., 1988. Coupling membranes as energy-transmitting cables. I. Filamentous mitochondria in fibroblasts and mitochondrial clusters in cardiomyocytes. J. Cell Biol. 107, 481–495.

Anderson, S., Bankier, A.T., Barrell, B.G., et al., 1981. Sequence and organization of the human mitochondrial genome. Nature 290, 457–465.

Bacman, S.R., Williams, S.L., Duan, D., et al., 2012. Manipulation of mtDNA heteroplasmy in all striated muscles of newborn mice by AAV9-mediated delivery of a mitochondria-targeted restriction endonuclease. Gene Ther. 19, 1101–1106.

Bayona-Bafaluy, M.P., Blits, B., Battersby, B.J., et al., 2005. Rapid directional shift of mitochondrial DNA heteroplasmy in animal tissues by a mitochondrially targeted restriction endonuclease. Proc. Natl. Acad. Sci. U.S.A. 102, 14392–14397.
Chen, H., Detmer, S.A., Ewald, A.J., et al., 2003. Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. J. Cell Biol. 160, 189–200.
Chinnery, P.F., Howell, N., Lightowlers, R.N., et al., 1997. Molecular pathology of MELAS and MERRF. The relationship between mutation load and clinical phenotypes. Brain J. Neurol. 120 (Pt 10), 1713–1721.
Cotán, D., Cordero, M.D., Garrido-Maraver, J., et al., 2011. Secondary coenzyme Q10 deficiency triggers mitochondria degradation by mitophagy in MELAS fibroblasts. FASEB J. 25, 2669–2687.
Crouser, E.D., Julian, M.W., Huff, J.E., et al., 2006. Carbamoyl phosphate synthase-1: a marker of mitochondrial damage and depletion in the liver during sepsis. Crit. Care Med. 34, 2439–2446.
Dai, Y., Zheng, K., Clark, J., et al., 2014. Rapamycin drives selection against a pathogenic heteroplasmic mitochondrial DNA mutation. Hum. Mol. Genet. 23, 637–647.
Debnath, J., Baehrecke, E.H., Kroemer, G., 2005. Does autophagy contribute to cell death? Autophagy 1, 66–74.
DiMauro, S., Hirano, M., 2009. Pathogenesis and treatment of mitochondrial disorders. In: Espinós, C., Felipo, V., Palau, F. (Eds.), Inherited Neuromuscular Diseases Springer, the Netherlands, pp. 139–170.
Ding, W.-X., Yin, X.-M., 2012. Mitophagy: mechanisms, pathophysiological roles, and analysis. Biol. Chem. 393, 547–564.
Finsterer, J., 2007. Genetic, pathogenetic, and phenotypic implications of the mitochondrial A3243G tRNALeu(UUR) mutation. Acta Neurol. Scand. 116, 1–14.
Gautier, C.A., Kitada, T., Shen, J., 2008. Loss of PINK1 causes mitochondrial functional defects and increased sensitivity to oxidative stress. Proc. Natl. Acad. Sci. U. S. A. 105, 11364–11369.
Gegg, M.E., Cooper, J.M., Chau, K.-Y., et al., 2010. Mitofusin 1 and mitofusin 2 are ubiquitinated in a PINK1/Parkin-dependent manner upon induction of mitophagy. Hum. Mol. Genet. 19, 4861–4870.
Geisler, S., Holmström, K.M., Skujat, D., et al., 2010. PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. Nat. Cell Biol. 12, 119–131.
Ghavami, S., Shojaei, S., Yeganeh, B., et al., 2014. Autophagy and apoptosis dysfunction in neurodegenerative disorders. Prog. Neurobiol. 112, 24–49.
Gilkerson, R.W., De Vries, R.L.A., Lebot, P., et al., 2012. Mitochondrial autophagy in cells with mtDNA mutations results from synergistic loss of transmembrane potential and mTORC1 inhibition. Hum. Mol. Genet. 21, 978–990.
Goldman, S.J., Taylor, R., Zhang, Y., et al., 2010. Autophagy and the degradation of mitochondria. Mitochondrion 10, 309–315.
Gomes, L.C., Scorrano, L., 2008. High levels of Fis1, a pro-fission mitochondrial protein, trigger autophagy. Biochim. Biophys. Acta 1777, 860–866.
Goto, Y., Nonaka, I., Horai, S., 1990. A mutation in the tRNA(Leu)(UUR) gene associated with the MELAS subgroup of mitochondrial encephalomyopathies. Nature 348, 651–653.
Hardie, D.G., Ross, F.A., Hawley, S.A., 2012. AMPK: a nutrient and energy sensor that maintains energy homeostasis. Nat. Rev. Mol. Cell Biol. 13, 251–262.
Hofmann, S., Bauer, MF., 2006. Mitochondrial disorders. In: eLS. John Wiley & Sons Ltd., Chichester, England.
Holt, I.J., Harding, A.E., Petty, R.K., et al., 1990. A new mitochondrial disease associated with mitochondrial DNA heteroplasmy. Am. J. Hum. Genet. 46, 428–433.
Howell, N., Bindoff, L.A., McCullough, D.A., et al., 1991. Leber hereditary optic neuropathy: identification of the same mitochondrial ND1 mutation in six pedigrees. Am. J. Hum. Genet. 49, 939–950.
Jing, C.-H., Wang, L., Liu, P.-P., et al., 2012. Autophagy activation is associated with neuroprotection against apoptosis via a mitochondrial pathway in a rat model of subarachnoid hemorrhage. Neuroscience 213, 144–153.
Kanki, T., Klionsky, D.J., 2008. Mitophagy in yeast occurs through a selective mechanism. J. Biol. Chem. 283, 32386–32393.
Kanki, T., Klionsky, D.J., 2010. The molecular mechanism of mitochondria autophagy in yeast. Mol. Microbiol. 75, 795–800.
Kanki, T., Wang, K., Cao, Y., et al., 2009. Atg32 is a mitochondrial protein that confers selectivity during mitophagy. Dev. Cell 17, 98–109.
Kim, I., Rodriguez-Enriquez, S., Lemasters, J.J., 2007. Minireview: selective degradation of mitochondria by mitophagy. Arch. Biochem. Biophys. 462, 245–253.

Klionsky, D.J., Cregg, J.M., Dunn, W.A., et al., 2003. A unified nomenclature for yeast autophagy-related genes. Dev. Cell 5, 539–545.
Lambert, A.J., Brand, M.D., 2009. Reactive oxygen species production by mitochondria. Methods Mol. Biol. 554, 165–181. Clifton NJ.
Lemasters, J.J., 2005. Selective mitochondrial autophagy, or mitophagy, as a targeted defense against oxidative stress, mitochondrial dysfunction, and aging. Rejuvenation Res. 8, 3–5.
Lemasters, J.J., Theruvath, T.P., Zhong, Z., et al., 2009. Mitochondrial calcium and the permeability transition in cell death. Biochim. Biophys. Acta 1787, 1395–1401.
Levine, B., Kroemer, G., 2008. Autophagy in the pathogenesis of disease. Cell 132, 27–42.
Li, Q., Zhang, T., Wang, J., et al., 2014. Rapamycin attenuates mitochondrial dysfunction via activation of mitophagy in experimental ischemic stroke. Biochem. Biophys. Res. Commun. 444, 182–188.
Liu, S., Sawada, T., Lee, S., et al., 2012. Parkinson's disease–associated kinase PINK1 regulates Miro protein level and axonal transport of mitochondria. PLoS Genet. 8 (3), e1002537.
López de Figueroa, P, Lotz, MK, Blanco, FJ, Caramés, B., 2015. Autophagy activation and protection from mitochondrial dysfunction in human chondrocytes. Arthritis Rheumatol. 67, 966–976.
Malena, A., Loro, E., Di Re, M., et al., 2009. Inhibition of mitochondrial fission favours mutant over wild-type mitochondrial DNA. Hum. Mol. Genet. 18, 3407–3416.
de la Mata, M., Garrido-Maraver, J., Cotán, D., et al., 2012. Recovery of MERRF fibroblasts and cybrids pathophysiology by coenzyme Q10. Neurotherapeutics 9, 446–463.
Matsuda, N., Sato, S., Shiba, K., et al., 2010. PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy. J. Cell Biol. 189, 211–221.
Maurer, I., Zierz, S., Möller, H.J., 2000. A selective defect of cytochrome c oxidase is present in brain of Alzheimer disease patients. Neurobiol. Aging 21, 455–462.
Merenlender-Wagner, A., Shemer, Z., Touloumi, O., et al., 2014. New horizons in schizophrenia treatment: autophagy protection is coupled with behavioral improvements in a mouse model of schizophrenia. Autophagy 10, 2324–2332.
Mizushima, N., 2007. Autophagy: process and function. Genes Dev. 21, 2861–2873.
Morán, M., Delmiro, A., Blázquez, A., et al., 2014. Bulk autophagy, but not mitophagy, is increased in cellular model of mitochondrial disease. Biochim. Biophys. Acta 1842, 1059–1070.
Moreira, P.I., Siedlak, S.L., Wang, X., et al., 2007. Increased autophagic degradation of mitochondria in Alzheimer disease. Autophagy 3, 614–615.
Narendra, D., Tanaka, A., Suen, D.-F., et al., 2008. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. J. Cell Biol. 183, 795–803.
Narendra, D.P., Youle, R.J., 2011. Targeting mitochondrial dysfunction: role for PINK1 and Parkin in mitochondrial quality control. Antioxid. Redox Signal. 14, 1929–1938.
Ni, H.-M., Williams, J.A., Ding, W.-X., 2014. Mitochondrial dynamics and mitochondrial quality control. Redox Biol. 4, 6–13.
Novak, I., Kirkin, V., McEwan, D.G., et al., 2010. Nix is a selective autophagy receptor for mitochondrial clearance. EMBO Rep. 11, 45–51.
Nunomura, A., Perry, G., Aliev, G., et al., 2001. Oxidative damage is the earliest event in Alzheimer disease. J. Neuropathol. Exp. Neurol. 60, 759–767.
Okamoto, K., Kondo-Okamoto, N., Ohsumi, Y., 2009. Mitochondria-anchored receptor Atg32 mediates degradation of mitochondria via selective autophagy. Dev. Cell 17, 87–97.
Palikaras, K., Tavernarakis, N., 2014. Mitochondrial homeostasis: the interplay between mitophagy and mitochondrial biogenesis. Exp. Gerontol. 56, 182–188.
Pan, T., Rawal, P., Wu, Y., et al., 2009. Rapamycin protects against rotenone-induced apoptosis through autophagy induction. Neuroscience 164, 541–551.
Parikh, S., Saneto, R., Falk, M.J., et al., 2009. A modern approach to the treatment of mitochondrial disease. Curr. Treat. Options Neurol. 11, 414–430.
Park, C.B., Larsson, N.-G., 2011. Mitochondrial DNA mutations in disease and aging. J. Cell Biol. 193, 809–818.
Piquereau, J., Godin, R., Deschênes, S., et al., 2013. Protective role of PARK2/Parkin in sepsis-induced cardiac contractile and mitochondrial dysfunction. Autophagy 9, 1837–1851.

Rakovic, A., Shurkewitsch, K., Seibler, P., et al., 2013. Phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1)-dependent ubiquitination of endogenous Parkin attenuates mitophagy. J. Biol. Chem. 288, 2223–2237.
Rasbach, K.A., Schnellmann, R.G., 2007. Signaling of mitochondrial biogenesis following oxidant injury. J. Biol. Chem. 282, 2355–2362.
Rodríguez-Hernández, A., Cordero, M.D., Salviati, L., et al., 2009. Coenzyme Q deficiency triggers mitochondria degradation by mitophagy. Autophagy 5, 19–32.
Rötig, A., Cormier, V., Blanche, S., et al., 1990. Pearson's marrow-pancreas syndrome. A multisystem mitochondrial disorder in infancy. J. Clin. Invest. 86, 1601–1608.
Sandoval, H., Thiagarajan, P., Dasgupta, S.K., et al., 2008. Essential role for Nix in autophagic maturation of erythroid cells. Nature 454, 232–235.
Santos, R.X., Correia, S.C., Wang, X., et al., 2010. A synergistic dysfunction of mitochondrial fission/fusion dynamics and mitophagy in Alzheimer's disease. J. Alzheimers Dis 20, S401–S412.
Saxton, W.M., Hollenbeck, P.J., 2012. The axonal transport of mitochondria. J. Cell Sci. 125, 2095–2104.
Schaefer, A.M., Taylor, R.W., Turnbull, D.M., et al., 2004. The epidemiology of mitochondrial disorders—past, present and future. Biochim. Biophys. Acta 1659, 115–120.
Scherz-Shouval, R., Shvets, E., Fass, E., et al., 2007. Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. EMBO J. 26, 1749–1760.
Shepherd, R.K., Checcarelli, N., Naini, A., et al., 2006. Measurement of ATP production in mitochondrial disorders. J. Inherit. Metab. Dis. 29, 86–91.
Shoffner, J.M., Lott, M.T., Lezza, A.M., et al., 1990. Myoclonic epilepsy and ragged-red fiber disease (MERRF) is associated with a mitochondrial DNA tRNA(Lys) mutation. Cell 61, 931–937.
Springer, W., Kahle, P.J., 2011. Regulation of PINK1-Parkin-mediated mitophagy. Autophagy 7, 266–278.
Sproule, D.M., Kaufmann, P., 2008. Mitochondrial encephalopathy, lactic acidosis, and strokelike episodes. Ann. N. Y. Acad. Sci. 1142, 133–158.
Suen, D.-F., Norris, K.L., Youle, R.J., 2008. Mitochondrial dynamics and apoptosis. Genes Dev. 22, 1577–1590.
Suen, D.-F., Narendra, D.P., Tanaka, A., et al., 2010. Parkin overexpression selects against a deleterious mtDNA mutation in heteroplasmic cybrid cells. Proc. Natl. Acad. Sci. U.S.A. 107, 11835–11840.
Taylor, R.W., Turnbull, D.M., 2005. Mitochondrial DNA mutations in human disease. Nat. Rev. Genet. 6, 389–402.
Tsukada, M., Ohsumi, Y., 1993. Isolation and characterization of autophagy-defective mutants of *Saccharomyces cerevisiae*. FEBS Lett. 333, 169–174.
Twig, G., Shirihai, O.S., 2011. The interplay between mitochondrial dynamics and mitophagy. Antioxid. Redox Signal. 14, 1939–1951.
Twig, G., Elorza, A., Molina, A.J.A., et al., 2008. Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. EMBO J. 27, 433–446.
Tyynismaa, H., Mjosund, K.P., Wanrooij, S., et al., 2005. Mutant mitochondrial helicase Twinkle causes multiple mtDNA deletions and a late-onset mitochondrial disease in mice. Proc. Natl. Acad. Sci. U.S.A. 102, 17687–17692.
Vives-Bauza, C., Przedborski, S., 2011. Mitophagy: the latest problem for Parkinson's disease. Trends Mol. Med. 17, 158–165.
Wallace, D.C., 1999. Mitochondrial diseases in man and mouse. Science 283, 1482–1488.
Wallace, D.C., Chalkia, D., 2013. Mitochondrial DNA genetics and the heteroplasmy conundrum in evolution and disease. Cold Spring Harb. Perspect. Biol. 5, a021220.
Wang, X., Winter, D., Ashrafi, G., et al., 2011. PINK1 and Parkin target Miro for phosphorylation and degradation to arrest mitochondrial motility. Cell 147, 893–906.
Wang, Y., Nartiss, Y., Steipe, B., et al., 2012. ROS-induced mitochondrial depolarization initiates PARK2/PARKIN-dependent mitochondrial degradation by autophagy. Autophagy 8, 1462–1476.
Yao, J., Irwin, R.W., Zhao, L., et al., 2009. Mitochondrial bioenergetic deficit precedes Alzheimer's pathology in female mouse model of Alzheimer's disease. Proc. Natl. Acad. Sci. U.S.A. 106, 14670–14675.
Youle, R.J., Narendra, D.P., 2011. Mechanisms of mitophagy. Nat. Rev. Mol. Cell Biol. 12, 9–14.
Youle, R.J., van der Bliek, A.M., 2012. Mitochondrial fission, fusion, and stress. Science 337, 1062–1065.
Yuan, J.-H., Sakiyama, Y., Higuchi, I., et al., 2013. Mitochondrial myopathy with autophagic vacuoles in patients with the m.8344A>G mutation. J. Clin. Pathol. 66, 659–664.

Zeviani, M., Moraes, C.T., DiMauro, S., et al., 1988. Deletions of mitochondrial DNA in Kearns–Sayre syndrome. Neurology 38, 1339–1346.
Zhang, H., Bosch-Marce, M., Shimoda, L.A., et al., 2008. Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia. J. Biol. Chem. 283, 10892–10903.
Zhang, Y., Sauler, M., Shinn, A.S., et al., 2014. Endothelial PINK1 mediates the protective effects of NLRP3 deficiency during lethal oxidant injury. J. Immunol. 192, 5296–5304. Baltim. Md 1950.
Züchner, S., Mersiyanova, I.V., Muglia, M., et al., 2004. Mutations in the mitochondrial GTPase mitofusin 2 cause Charcot–Marie–Tooth neuropathy type 2A. Nat. Genet. 36, 449–451.

CHAPTER

9

Autophagy Regulation by HMGB1 in Disease

Rui Kang and Daolin Tang

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Abstract

Autophagy is an evolutionarily-conserved catabolic process that degrades cytoplasmic material in the lysosome via a complicated pathway. Functional autophagic pathways are crucial for embryonic development, tissue differentiation, and cell survival. In contrast, the dysregulation of autophagy is involved in multiple pathological conditions including inflammation, immunity, metabolism, and death. Accumulating evidence indicates that high-mobility group box 1 (HMGB1), the best-characterized nonhistone chromosomal protein, and damage-associated molecular pattern molecule can regulate autophagy in a localization-dependent manner. Nuclear HMGB1 regulates heat shock protein β-1 expression to control mitochondrial quality by mitophagy. Cytoplasmic HMGB1 acts as a Belcin-1-binding protein to induce autophagosome formation. Extracellular HMGB1 can bind the receptor for advanced glycation endproducts to induce class III

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phosphatidylinositol 3-kinase core complex formation in autophagy. In addition, transcriptional and post-translational regulation of HMGB1 by miRNA34A, miRNA22, miR-let-7f-1, and poly-ADP-ribosylation can fine-tune HMGB1-mediated autophagy. Knockout of HMGB1 *in vitro* and *in vivo* limits autophagy that contributes to infection and sterile inflammation. This chapter focuses on the structure and function of HMGB1 as an important regulator of autophagy, as well as its role in cancer therapy, inflammation, immunity, and the central nervous system.

INTRODUCTION

Christian de Duve first coined the word "autophagy" in 1963. Since then, significant progress has been made toward understanding the process and function of autophagy; autophagy has developed into an entire field of study with critical roles in biology and medicine. Autophagy is divided into three general types: chaperone-mediated autophagy, microautophagy, and macroautophagy (hereafter referred to as autophagy). As a cellular self-digestion process, autophagy is involved in the degradation of proteins, organelles, and invading pathogens via step-by-step formation of various membrane vesicles (e.g., phagophores, autophagosomes, and autolysosomes). Currently, 38 autophagy-related genes (ATGs) have been identified to form many functional complexes to control this complicated membrane dynamic in organisms ranging from yeasts to mammalian cells. Functional autophagic pathways are crucial for embryonic development, tissue differentiation, and cell survival. In contrast, the dysregulation of autophagy is involved in multiple pathological conditions, including inflammation, immunity, metabolism, and death. Thus, autophagy can promote both cell survival and cell death, depending on the specific conditions and cell/tissue type (Green and Levine, 2014).

HMGB1, a member of the superfamily of high-mobility group box (HMGB) proteins, was first identified in 1973. As an evolutionarily-conserved, nonhistone protein, HMGB1 is expressed in most tissues and normally locates in the nucleus via two lysine-rich nuclear localization sequences. HMGB1 is composed of two DNA-binding domains (box A and B) and a long acidic tail at the C-terminus. In the nucleus, HMGB1 plays a critical role, with DNA chaperone activity in the maintenance of chromosome structure and functions such as genomic stability, DNA damage response, gene transcription, V(D)J recombination, and nucleosome dynamics. Loss of HMGB1 in cells and tissues will lead to nuclear catastrophe and proinflammatory nucleosomal release. Cytosolic HMGB1 expression is increased in response to stress and promotes cell survival via autophagy. In addition, HMGB1 can be passively released by dead, dying, or injured cells or actively secreted by immune or epithelial cells. As a damage-associated molecular pattern (DAMP), extracellular HMGB1 binds cell surface or cytosolic receptors such as receptor for advanced glycation endproducts (RAGE) and toll-like receptors (TLRs, e.g., TLR2, TLR4, and TLR9) to initiate signals that produce cytokines or chemokines to link cell death and immunity. Alternatively, extracellular HMGB1 can be taken up directly to promote inflammation and metabolism in immune and cancer cells, respectively. Both intracellular and extracellular HMGB1 are implicated in human health and disease (Kang et al., 2014a). Indeed, HMGB1 is an emerging therapeutic target in inflammation-associated diseases including sterile inflammation, infection, and cancer.

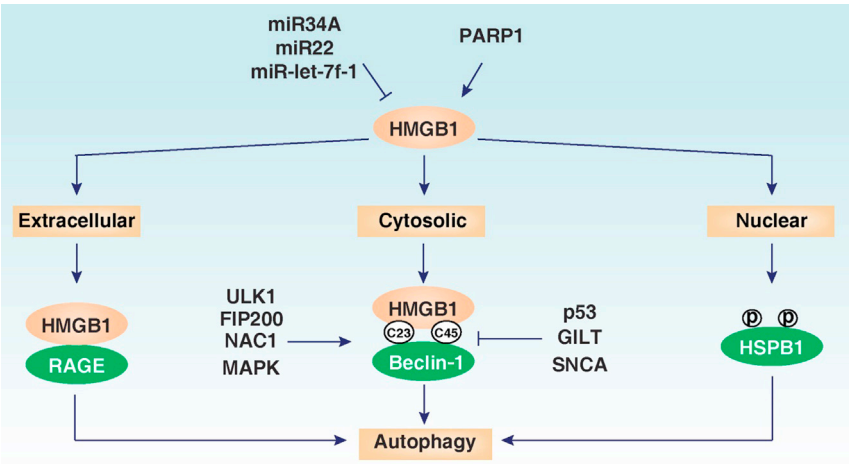

FIGURE 9.1 Regulation mechanisms and signaling pathways of HMGB1-mediated autophagy.

In addition to ATG proteins, many non-ATG proteins, including HMGB1, are now known to regulate the process of autophagy. Deletion, depletion, or inhibition of HMGB1 in many cells markedly diminishes microtubule-associated protein 1 light chain 3 (LC3) punctae and autophagosome formation associated with increased expression of p62/SQSTM1 in cells following treatment with various autophagic stimuli (Tang et al., 2010b). HMGB1 has location-dependent proautophagic activities and is fine-tuned and regulated at transcriptional and posttranslational levels (Figure 9.1). This chapter focuses on the structure and function of HMGB1 as a regulator of autophagy in disease.

NUCLEAR HMGB1-MEDIATED AUTOPHAGY

Mitochondria are the powerhouses of the cell and are involved in the central regulation of energy production, metabolism, and cell death. As an important quality control system, mitophagy is the process of selective degradation of defective mitochondria via autophagy in response to damage or stress. Impaired mitophagy may cause oxidative stress, inflammatory response, and cell death. A recent study indicates that nuclear HMGB1 sustains mitochondrial morphology and function by transcriptional regulation of heat shock protein β-1 (HSPB1) expression (Tang et al., 2011a).

The production of adenosine triphosphate (ATP) from mitochondria is reduced in HMGB1-deficient cells. This process is associated with decreased glycolysis or oxidative phosphorylation in HMGB1-deficient cells, suggesting a role of HMGB1 in the regulation of mitochondrial respiration (Tang et al., 2011a). Mitochondrial morphology is controlled by mitochondrial fission and fusion and both contribute to maintenance of mitochondrial function. Most mitochondria in normal cells exhibit long and tubular morphology, while mitochondria become significantly shorter, rounder, and fragmented when HMGB1 is deficient in mouse embryonic fibroblasts (MEFs) and cancer cells. Interestingly, the key mediators of

mitochondrial fusion and fission do not significantly differ in HMGB1$^{-/-}$ MEFs. In contrast, the protein and mRNA expression of HSPB1 (also known as HSP25 in mice and HSP27 in humans) is dramatically reduced in HMGB1 knockout and knockdown cells, although the precise molecular mechanism is unknown. Like HMGB1 deficiency, suppression of HSP27 expression by specific shRNA also inhibits ATP production, mitochondrial respiration, and mitochondrial fragmentation in MEFs and cancer cells (Tang et al., 2011a). Importantly, forced expression of HSP27 by gene transfection in HMGB1-deficient cells corrects the deficiency in mitochondrial respiration, ATP production, and mitochondrial fragmentation, which is observed in HMGB1-deficient cells. These findings suggest that HSPB1 is the mediator of HMGB1's effect on the regulation of mitochondrial function and morphology.

The HMGB1–HSPB1 pathway regulates mitophagy via cytoskeletal alterations, which is important for autophagic dynamics, including the process of autophagosome–lysosome fusion. Previous studies indicate that HSPB1 has a direct influence on the dynamics of cytoskeletal elements by HSPB1 phosphorylation. Similarly, by using cytoskeleton inhibitor cytochalasin D, loss of HSPB1 or mutation of its phosphorylation sites at serines 15 and 86 decreases starvation and rotenone-induced autophagy and mitophagy and impairs autophagosome and lysosome fusion (Tang et al., 2011a).

Upon mitochondrial membrane depolarization, PTEN-induced putative kinase-1 (Pink1) accumulates in the outer mitochondrial membrane and mediates mitochondrial translocation of Parkin (a gene implicated in autosomal recessive juvenile Parkinsonism) following injury, especially oxidative stress (Geisler et al., 2010). Subsequently, mitochondrial Parkin drives the formation of Lys27-linked ubiquitin chains on the outer membrane of voltage-dependent anion channel 1 (VDAC1) (Geisler et al., 2010). The Parkin translocation and VDAC1 ubiquitination during mitochondrial depolarization is limited after inhibition of the HMGB1–HSPB1 pathway. Moreover, knockdown of Pink1 or Parkin abolishes HSPB1-induced restoration in ATP production and reduction in mitochondrial fragmentation in HMGB1-deficient cells, suggesting that Pink1 and Parkin contribute to HSPB1/HMGB1-mediated mitophagy (Tang et al., 2011a). In addition to regulating the cytoskeleton, HMGB1 and HSPB1 translocate into the mitochondria during cellular stress and may play a direct autophagy-independent role in protecting mitochondrial quality. For example, a recent study indicated that the mitochondrial HMGB1-mediated DNA damage response is autophagy-independent (Ito et al., 2014).

In addition to HSPB1, nuclear HMGB1 can regulate p53 localization in colon cancer cells (Livesey et al., 2012). p53, a tumor suppressor, regulates autophagy through both transcription-dependent and -independent mechanisms. Cytosolic p53 inhibits autophagy through a transcription-independent mechanism (Tasdemir et al., 2008), whereas nuclear p53 promotes autophagy through a transcription-dependent mechanism (Crighton et al., 2006; Gao et al., 2011). In the nucleus, HMGB1 and p53 form a complex that regulates the cytoplasmic localization of the reciprocal binding protein and subsequent levels of autophagy (Livesey et al., 2012). Loss of nuclear HMGB1 increases p53 translocation from the nucleus to the cytosol and then inhibits autophagy and increases apoptosis. In contrast, loss of nuclear p53 increases HMGB1 translocation from the nucleus to the cytosol and then induces autophagy and inhibits apoptosis. Thus, HMGB1 and p53 play a role in the cross-regulation of apoptosis and autophagy.

CYTOSOLIC HMGB1-MEDIATED AUTOPHAGY

Translocation of HMGB1 to the cytoplasm and extracellular space occurs following several stressors, including induction of necrosis, apoptosis, and autophagy. For example, classical autophagic stimuli such as rapamycin or starvation can promote HMGB1 translocation at the early stage, which is not associated with lactate dehydrogenase release, a marker of necrosis (Tang et al., 2010b). The antioxidant *N*-acetyl cysteine inhibits, while knockdown of antioxidant enzyme superoxide dismutase 1 (SOD1) promotes HMGB1 cytosolic translocation in autophagy (Tang et al., 2011b). Thus, reactive oxygen species (ROS) signals are required for cytoplasmic translocation of HMGB1 in autophagy.

To assess the role of cytoplasmic HMGB1 in the setting of autophagy, cytoplasts (anucleate cells) are produced. In response to starvation, $HMGB1^{-/-}$ MEF cytoplasts have a lower level of LC3 punctae than $HMGB1^{+/+}$ cytoplasts, indicating that cytoplasmic HMGB1 is required for starvation-stimulated autophagy (Tang et al., 2010b). Functionally, cytosolic HMGB1 is a Beclin-1-binding protein to induce autophagy. Beclin-1, the mammalian orthologue of yeast Atg6, plays a central role in the regulation of autophagosome formation by interacting with several cofactors (e.g., Atg14L, UVRAG, bif-1, rubicon, ambra1, barkor, HMGB1, nPIST, VMP1, SLAM, IP_3R, Pink1, and survivin) to activate and form class III phosphatidylinositol 3-kinase (PI3KC3) core complex (Kang et al., 2011b). Interestingly, HMGB1 and Beclin-1 may have the same origin because these two molecules share considerable sequences in yeast and mammalian cells. In addition to directly binding to Beclin-1, HMGB1 also promotes the phosphorylation of bcl-2, an autophagic inhibitor, by activation of the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway following starvation. This blocks the interaction between Beclin-1 and bcl-2 (Tang et al., 2010b).

The translocation and activity of HMGB1 is regulated by its redox status. Human HMGB1 contains three cysteines at the sites of 23, 45 (A box), and 106 (B box). Under mild oxidative conditions, the vicinal cysteines C23 and C45 readily form an intramolecular disulfide bridge, whereas C106 remains in a reduced form. Mutation of C106 to serine (C106S) leads to HMGB1 translocation to the cytosol and enhances the interaction with Beclin-1 (Tang et al., 2010b). Meanwhile, mutation of both C23 and C45 to serine (C23S and C45S) impairs the interaction between HMGB1 and Belcin-1 and therefore cannot disrupt the interaction between Belcin-1 and bcl-2. Thus, oxidation of HMGB1 regulates its localization and ability to sustain autophagy.

In addition to the redox state of HMGB1, several proteins have been demonstrated to positively or negatively regulate HMGB1–Beclin-1 complex formation. Unc-51-like autophagy-activating kinase 1 (ULK1), the mammalian orthologue of yeast Atg1, is a serine/threonine-protein kinase involved in the formation of ULK1-ATG13-FIP200 complex in the induction of autophagy in response to starvation. Knockdown of HMGB1 does not affect the formation of ULK1-ATG13-FIP200 complex in anticancer agent-induced autophagy. In contrast, knockdown of ULK1 or FIP200 inhibits the interaction between HMGB1 and Beclin-1 in osteosarcoma cells (Huang et al., 2012) or in murine pseudomonas infection (Li et al., 2014b). Thus, HMGB1 is a downstream signal from ULK1-ATG13-FIP200 complex formation and facilitates autophagy by interacting with Beclin-1. Nucleus accumbens-1 (NAC1) is normally a nuclear protein belonging to the BTB/POZ gene family.

Upon stress, NAC1 can bind to HMGB1 and increase the translocation of HMGB1 from the nucleus to the cytoplasm and subsequent autophagy by promotion of HMGB1–Beclin-1 complex formation (Zhang et al., 2012).

In addition to p53 as discussed above, SNCA/α-synuclein (Song et al., 2014) and gamma-interferon inducible lysosomal thiol reductase (GILT) (Chiang and Maric, 2011) negatively regulate autophagy by disrupting the interaction between HMGB1 and Beclin-1. SNCA is a presynaptic neuronal protein that is linked genetically and neuropathologically to Parkinson's disease (PD). Unlike NAC1, SNCA binds to HMGB1, which inhibits the cytosolic translocation of HMGB1 and impairs HMGB1–Beclin-1 binding (Song et al., 2014). Thus, SNCA-induced impairment of autophagy by HMGB1 may be implicated in the development of PD. GILT, a lysosomal enzyme, can reduce protein disulfide bonds. Loss of GILT in fibroblasts increases oxidative stress and the cytosolic translocation of HMGB1, which promotes HMGB1–Beclin-1 binding and autophagy (Chiang and Maric, 2011).

EXTRACELLULAR HMGB1-MEDIATED AUTOPHAGY

Besides its intracellular role, HMGB1 acts as an extracellular DAMP with cytokine, chemokine, and growth factor activity. The redox status of HMGB1 not only determines the immune activity of HMGB1, but also regulates its autophagic activity. The reduced HMGB1 decreases the effectiveness of many anticancer agents such as oxaliplatin, melphalan, adriamycin, paclitaxel, and ultraviolet light (Tang et al., 2010a). In contrast, the oxidized HMGB1 increases caspase activity and apoptosis following anticancer treatment (Tang et al., 2010a). This suggests that the redox status of HMGB1 in the tumor microenvironment modifies the anticancer activity of chemotherapy and radiotherapy.

Extracellular HMGB1 can stimulate cells following binding to RAGE and TLRs. TLR4 is required for HMGB1-mediated cytokine release in macrophages (Yang et al., 2010), whereas RAGE is required for HMGB1-mediated cell migration in fibroblasts (Palumbo et al., 2004). Interestingly, knockdown of RAGE, but not TLR4, in cancer cells inhibits reduced HMGB1-induced PI3KC3 complex formation and autophagy (Tang et al., 2010a). RAGE is a multiligand MHC Class III receptor of the immunoglobulin gene superfamily. The interaction between RAGE and its ligands, including HMGB1, promotes pro-inflammatory signal pathway activation and is implicated in the pathogenesis of aging, neurodegeneration, cardiovascular disease, cancer, and diabetes. RAGE is upregulated in several cancers including pancreatic cancer and contributes to drug resistance and tumorigenesis by maintenance of autophagy and limitation of apoptosis (Kang et al., 2010, 2011a). Thus, blocking the HMGB1–RAGE pathway can slow tumor growth and prevent cancer initiation. In addition, HMGB1–RAGE pathway mediated autophagy is required for the formation of neutrophil extracellular traps (Maugeri et al., 2014).

TRANSCRIPTIONAL REGULATION OF HMGB1 IN AUTOPHAGY

HMGB1 gene expression is regulated by transcription factors and microRNAs (miRNAs) in stress. Currently, specific miRNA, but not transcription factor, is identified to perform

transcriptional regulation of HMGB1 in autophagy. As a class of posttranscriptional regulators, miRNAs are small, noncoding RNA molecules that bind to complementary sequences in the 3′ untranslated region (3′UTR) of multiple target messenger RNAs (mRNAs). They are conserved across species and expressed in a tissue-specific and developmental stage–specific manner. Alterations in the expression of several miRNAs are involved in most biological processes and have been implicated in many diseases by silencing or promoting gene expression.

miR34A, a tumor suppressor, inhibits HMGB1 expression in retinoblastoma cells and leads to a decrease in autophagy following starvation and chemotherapy treatment (Liu et al., 2014a). miR34A is widely expressed and regulates a number of target genes that are involved in cell cycle, proliferation, death, and differentiation. miR34A mimic decreases the mRNA levels of HMGB1, whereas antagomiR34A increases HMGB1 mRNA expression in human retinoblastoma cells, suggesting that HMGB1 may be a direct target of miR34A (Liu et al., 2014a). The mRNA expression of HMGB1 is upregulated at 1–2 h following starvation-induced autophagy, and then returns to the baseline levels at 6 h when miR34A expression is increased in the retinoblastoma cell. Similarly, with knockdown of HMGB1, miR34A mimic can inhibit starvation- and anticancer agent–induced autophagy and enhance chemotherapy sensitivity. Moreover, forced expression of HMGB1 by transfection with HMGB1 cDNA restores starvation-induced autophagy following miR34A mimic treatment. These findings suggest that miR34A is an inhibitor of autophagy by downregulation of HMGB1 expression. In addition to HMGB1, miR34A can suppress SIRT1 expression and knockdown of SIRT1 inhibits autophagy in several cell types (Lee et al., 2008; Yamakuchi et al., 2008), suggesting that miR34A regulates autophagy by targeting different genes.

Another study suggests that miR22 inhibits HMGB1 expression by binding the 3′ UTR of HMGB1 in osteosarcoma cells (Li et al., 2014a). miR22 was originally identified in cervical cancer cell lines, but was later found to be ubiquitously expressed in various tissues and cancer cells. miR22-mediated transcriptional regulation of HMGB1 inhibits autophagy and chemotherapy resistance in osteosarcoma cells (Li et al., 2014a). In addition, HMGB1 is a direct target of miR-let-7f-1 and forced expression of miR-let-7f-1 suppresses HMGB1 expression and autophagy in medulloblastoma cells (Pannuru et al., 2014). In addition to miR34A/miR22/miR-let-7f-1, several miRNAs (e.g., miR218, miR181, and miR1192) have been demonstrated to function as negative regulators of HMGB1 gene expression by directly targeting its 3′ UTR in various cells. It is unclear whether these miRNAs are involved in transcriptional regulation of HMGB1 expression in autophagy.

POSTTRANSLATIONAL MODIFICATION OF HMGB1 IN AUTOPHAGY

Posttranslational modification (PTM) plays a critical role in the regulation of proteins' structure, location, and activity in multiple cell processes. Cumulative evidence confirms that multiple PTMs exist in ATG (e.g., ULK1, Belcin-1, and LC3) and non-ATG proteins (e.g., HMGB1, AMPK, and PI3KC3) during complicated membrane dynamics in autophagy (Xie et al., 2015). Following stress, the HMGB1 protein is modified by different PTMs such as acetylation, poly-ADP-ribosylation, phosphorylation, and oxidation, which regulates

HMGB1 release and activity. In addition to oxidation of HMGB1 directly affecting HMGB1–Beclin-1 complex formation, poly-ADP-ribosylation of HMGB1 may regulate autophagy in cancer cells (Yang et al., 2015). The addition to proteins of the negatively charged polymer of ADP-ribose (PAR), which is synthesized by PAR polymerases (PARPs) from NAD+, is a unique PTM. Poly-ADP-ribosylation of HMGB1 is specifically mediated by PARP1 in cancer cells, which in turn contributes to cytoplasmic translocation of HMGB1 and subsequent HMGB1–Beclin-1 complex formation following TNF-related apoptosis-inducing ligand (TRAIL) treatment (Yang et al., 2015). Moreover, poly-ADP-ribosylation of HMGB1 is increased when HMGB1 C106 is mutated, suggesting an interplay between different PTMs for control of HMGB1 translocation and activity in autophagy. PARP1-mediated poly-ADP-ribosylation of HMGB1 plays a cytoprotective role in TRAIL-mediated cell death. Genetic and pharmacological inhibition of the PARP1–HMGB1 autophagy pathway enhances TRAIL-mediated cell death *in vitro* and *in vivo* by inhibition of caspase 8 activity (Yang et al., 2015). Caspase 8 is a critical effector in the death receptor–mediated apoptosis pathway. Interestingly, PARP1 may promote HMGB1 acetylation and release in immune cells (Yang et al., 2014). Thus, different PTMs may cooperatively regulate HMGB1 release and activity in autophagy, although the details remain unknown.

HMGB1-MEDIATED AUTOPHAGY IN CANCER THERAPY

Cancer treatment includes surgery and nonsurgical treatment; chemotherapy is the most common type of nonsurgical treatment. However, intrinsic and acquired drug resistance is a major challenge for most chemotherapeutic agents in tumor therapy. Many mechanisms, including autophagy, have been reported to be responsible for chemoresistance. Both autophagy and apoptosis can be triggered and regulated by common signals and proteins in chemotherapy. In many cases, inhibition of autophagy increases chemosensitivity, whereas induction of autophagy promotes chemoresistance. One of the important mechanisms of autophagy-mediated chemoresistance is that autophagy can degrade apoptotic effectors such as caspases 8 and 9. In addition, the autophagy-independent role of ATG also contributes to inhibition of caspase activity by protein–protein interaction. Of note, increased caspase activity can promote cleavage of ATG such as Beclin-1, suggesting a complicated and time-dependent interplay between autophagy and apoptosis.

A number of studies have demonstrated that knockdown of HMGB1 expression by RNAi (e.g., shRNA and siRNA) in several cancer cells inhibits autophagy and enhances the anticancer activity of cytotoxic agents (Amornsupak et al., 2014; Huang et al., 2012; Liu et al., 2014a,b; Pan et al., 2014; Su et al., 2015; Yang et al., 2012, 2015; Zhang et al., 2012; Zhao et al., 2011). In contrast, overexpression of HMGB1 expression by gene transfection promotes autophagy and inhibits drug sensitivity (Huang et al., 2012; Liu et al., 2011; Livesey et al., 2012). In addition to intracellular HMGB1-mediated autophagy in drug resistance, HMGB1 release in chemotherapy also promotes autophagy and drug resistance (Liu et al., 2011; Tang et al., 2010a). These findings suggest that HMGB1 is a potential target for cancer therapy.

HMGB1-MEDIATED AUTOPHAGY IN INFLAMMATION

Inflammation is a powerful homeostatic mechanism involved in host defense and tissue repair and remodeling. During infections from pathogens or sterile inflammation from tissue injury, a cascade of signals results in the recruitment and activation of inflammatory cells such as neutrophils and macrophages. These cells can produce and secrete a number of cytokines and chemokines that trigger the activation of immune responses. This process is involved in the activation of multiple proinflammatory signaling pathways (e.g., NF-κB and inflammasome) and upregulated autophagy (Tang et al., 2012). In particular, a number of studies demonstrate that upregulation of autophagy can inhibit activation of inflammasome by various mechanisms such as limiting mitochondrial ROS production or promoting the degradation of core components (e.g., NALP3) of inflammasome (Shi et al., 2012; Zhou et al., 2011). Thus, autophagy-deficient mice are more sensitive to sepsis, inflammatory bowel disorder, and other inflammatory diseases. In contrast, a selective type of autophagy termed "xenophagy" can directly digest invading pathogen and prevent infection (Bauckman et al., 2015). These findings suggest a dual role of autophagy in inflammation, depending on the context.

HMGB1 is a critical mediator of infection and sterile inflammation. On the one hand, autophagy promotes HMGB1's release, translocation, and degradation in inflammation (Zhang et al., 2013). On the other hand, loss of HMGB1 in immune cells or tissue can impair the autophagic response and accelerate the inflammatory response. For example, *in vivo* conditional knockout of HMGB1 in myeloid and intestinal epithelial cells causes autophagy deficiency and increases inflammasome activation and animal death in response to endotoxemia, bacterial infection, and inflammatory bowel disease (Yanai et al., 2013; Zhu et al., 2015). In addition, knockout of HMGB1 in the liver and pancreas accelerates the sterile inflammatory response with mitochondrial injury and proinflammatory nucleosome release in animal models of ischemia–reperfusion and pancreatitis, respectively (Huang et al., 2014; Kang et al., 2014b). Therefore, these studies reveal a unique function of HMGB1-mediated autophagy in the inflammatory response *in vivo*. More recently, HMGB1–DNA complex induced autophagy was found to limit AIM2 inflammasome activation and cytokine IL-1β release through RAGE, suggesting a novel negative feedback regulatory mechanism for controlling HMGB1 activity in the innate immune response (Liu et al., 2014b).

HMGB1-MEDIATED AUTOPHAGY IN IMMUNITY

Autophagy plays a crucial role in adaptive and innate immunity; the term "immunophagy" (Deretic, 2006) refers to all such processes collectively. Autophagy and ATG proteins participate in the regulation of survival, differentiation, and function of immune cells including monocytes, dendritic cells (DCs), and NK, B, and T cells. Thus, the systemic stimulation or suppression of autophagy can modulate immune responses in physiological and pathological brain processes. Similarly, with knockout of HMGB1 in fibroblasts,

conditional knockout of HMGB1 in DCs and NK cells in mice impairs the metabolism response and ATP production. In addition, HMGB1 and Beclin-1 are coexpressed in the invading T cells in the muscle tissue of myositis patients, which contributes to T-cell survival (Zong et al., 2014). The function of HMGB1-mediated autophagy in immune cells needs further exploration.

HMGB1-MEDIATED AUTOPHAGY IN THE CENTRAL NERVOUS SYSTEM

Protein misfolding and aggregation is the common cause and pathological mechanism of neurodegenerative diseases such as Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), PD, and Huntington's disease (HD). Autophagy can selectively remove abnormally folded proteins via the lysosomal pathway. Thus, pharmacological manipulation of autophagy may have neuroprotective effects in certain neurodegenerative diseases. HMGB1 expressed in the central nervous system and dysfunction of HMGB1 are implicated in AD, ALS, PD, and HD (Kang et al., 2014a). However, exploration of the role of HMGB1-mediated autophagy in neurodegenerative diseases has only begun very recently. HD is a progressive brain disorder caused by polyglutamine (polyQ) accumulation. Downregulation of HMGB1 expression is observed when mutant polyQ proteins are expressed in HD (Qi et al., 2007). HMGB1 can directly bind to polyQ aggregates and then promote degradation by chaperone-mediated autophagy (Min et al., 2013). Thus, loss of HMGB1-mediated autophagy can increase protein misfolding and aggregation in HD. SNCA and its rare mutations are responsible for PD pathogenesis. SNCA inhibits autophagy by disrupting the interaction between HMGB1 and Belcin-1. Thus, HMGB1 may be a new target for drug intervention to restore the deficient autophagy caused by SNCA. In addition, HMGB1–Beclin-1 complex formation can protect against neuronal toxicity by induction of autophagy in rat cortical neurons (Perez-Carrion and Cena, 2013).

CONCLUSIONS

HMGB1 is a conserved protein originally identified as a nuclear DNA–binding protein and a new potent autophagic regulator in response to a broad array of cellular damage. Autophagy is a complex process that proceeds through several ATG protein complexes, each of which can be regulated at multiple levels. HMGB1 plays transcription-dependent and -independent roles in the induction of autophagy. The activity of HMGB1 in autophagy is also regulated by miRNA, PTMs, and protein–protein interaction. Besides HMGB1-dependent autophagy, HMGB1-independent autophagy has also recently been demonstrated, although the mechanism remains unknown (Sun and Tang, 2014). Indeed, HMGB1 plays a tissue- and cell-specific role in the regulation of the stress response (Tang et al., 2014). Understanding the structure and function of HMGB1 will provide further insight into the autophagy interaction network and guide autophagy-based therapeutic interventions in human diseases.

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References

Amornsupak, K., Insawang, T., Thuwajit, P., et al., 2014. Cancer-associated fibroblasts induce high mobility group box 1 and contribute to resistance to doxorubicin in breast cancer cells. BMC Cancer 14, 955.

Bauckman, K.A., Owusu-Boaitey, N., Mysorekar, I.U. 2015. Selective autophagy: xenophagy. Methods 75, 120–127.

Chiang, H.S., Maric, M., 2011. Lysosomal thiol reductase negatively regulates autophagy by altering glutathione synthesis and oxidation. Free Radic. Biol. Med. 51, 688–699.

Crighton, D., Wilkinson, S., O'Prey, J., et al., 2006. DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. Cell 126, 121–134.

Deretic, V., 2006. Autophagy as an immune defense mechanism. Curr. Opin. Immunol. 18, 375–382.

Gao, W., Shen, Z., Shang, L., et al., 2011. Upregulation of human autophagy-initiation kinase ULK1 by tumor suppressor p53 contributes to DNA-damage-induced cell death. Cell Death Differ. 18, 1598–1607.

Geisler, S., Holmstrom, K.M., Skujat, D., et al., 2010. PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. Nat. Cell Biol. 12, 119–131.

Green, D.R., Levine, B., 2014. To be or not to be? How selective autophagy and cell death govern cell fate. Cell 157, 65–75.

Huang, H., Nace, G.W., McDonald, K.A., et al., 2014. Hepatocyte-specific high-mobility group box 1 deletion worsens the injury in liver ischemia/reperfusion: a role for intracellular high-mobility group box 1 in cellular protection. Hepatology 59, 1984–1997.

Huang, J., Ni, J., Liu, K., et al., 2012. HMGB1 promotes drug resistance in osteosarcoma. Cancer Res. 72, 230–238.

Ito, H., Fujita, K., Tagawa, K., et al., 2014. HMGB1 facilitates repair of mitochondrial DNA damage and extends the lifespan of mutant ataxin-1 knock-in mice. EMBO Mol. Med. 7, 78–101.

Kang, R., Tang, D., Schapiro, N.E., et al., 2010. The receptor for advanced glycation end products (RAGE) sustains autophagy and limits apoptosis, promoting pancreatic tumor cell survival. Cell Death Differ. 17, 666–676.

Kang, R., Tang, D., Loze, M.T., et al., 2011a. Apoptosis to autophagy switch triggered by the MHC class III-encoded receptor for advanced glycation endproducts (RAGE). Autophagy 7, 91–93.

Kang, R., Zeh, H.J., Lotze, M.T., et al., 2011b. The Beclin 1 network regulates autophagy and apoptosis. Cell Death Differ. 18, 571–580.

Kang, R., Chen, R., Zhang, Q., et al., 2014a. HMGB1 in health and disease. Mol. Aspects Med. 40, 1–116.

Kang, R., Zhang, Q., Hou, W., et al., 2014b. Intracellular Hmgb1 inhibits inflammatory nucleosome release and limits acute pancreatitis in mice. Gastroenterology 146, 1097–1107.

Lee, I.H., Cao, L., Mostoslavsky, R., et al., 2008. A role for the NAD-dependent deacetylase Sirt1 in the regulation of autophagy. Proc. Natl. Acad. Sci. U.S.A. 105, 3374–3379.

Li, X., Wang, S., Chen, Y., et al., 2014a. miR-22 targets the 3′ UTR of HMGB1 and inhibits the HMGB1-associated autophagy in osteosarcoma cells during chemotherapy. Tumour Biol. 35, 6021–6028.

Li, Y., Gan, C.P., Zhang, S., et al., 2014b. FIP200 is involved in murine pseudomonas infection by regulating HMGB1 intracellular translocation. Cell. Physiol. Biochem. 33, 1733–1744.

Liu, K., Huang, J., Xie, M., et al., 2014a. MIR34A regulates autophagy and apoptosis by targeting HMGB1 in the retinoblastoma cell. Autophagy 10, 442–452.

Liu, L., Yang, M., Kang, R., et al., 2011. HMGB1-induced autophagy promotes chemotherapy resistance in leukemia cells. Leukemia 25, 23–31.

Liu, L., Yang, M., Kang, R., et al., 2014b. HMGB1-DNA complex-induced autophagy limits AIM2 inflammasome activation through RAGE. Biochem. Biophys. Res. Commun. 450, 851–856.

Liu, Y., Zhao, L., Ju, Y., et al., 2014c. A novel androstenedione derivative induces ROS-mediated autophagy and attenuates drug resistance in osteosarcoma by inhibiting macrophage migration inhibitory factor (MIF). Cell Death Dis. 5, e1361.

Livesey, K.M., Kang, R., Vernon, P., et al., 2012. p53/HMGB1 complexes regulate autophagy and apoptosis. Cancer Res. 72, 1996–2005.
Maugeri, N., Campana, L., Gavina, M., et al., 2014. Activated platelets present high mobility group box 1 to neutrophils, inducing autophagy and promoting the extrusion of neutrophil extracellular traps. J. Thromb. Haemost. 12, 2074–2088.
Min, H.J., Ko, E.A., Wu, J., et al., 2013. Chaperone-like activity of high-mobility group box 1 protein and its role in reducing the formation of polyglutamine aggregates. J. Immunol. 190, 1797–1806.
Palumbo, R., Sampaolesi, M., De Marchis, F., et al., 2004. Extracellular HMGB1, a signal of tissue damage, induces mesoangioblast migration and proliferation. J. Cell Biol. 164, 441–449.
Pan, B., Chen, D., Huang, J., et al., 2014. HMGB1-mediated autophagy promotes docetaxel resistance in human lung adenocarcinoma. Mol. Cancer 13, 165.
Pannuru, P., Dontula, R., Khan, A.A., et al., 2014. miR-let-7f-1 regulates SPARC mediated cisplatin resistance in medulloblastoma cells. Cell Signal. 26, 2193–2201.
Perez-Carrion, M.D., Cena, V., 2013. Knocking down HMGB1 using dendrimer-delivered siRNA unveils its key role in NMDA-induced autophagy in rat cortical neurons. Pharm Res. 30, 2584–2595.
Qi, M.L., Tagawa, K., Enokido, Y., et al., 2007. Proteome analysis of soluble nuclear proteins reveals that HMGB1/2 suppress genotoxic stress in polyglutamine diseases. Nat. Cell Biol. 9, 402–414.
Shi, C.S., Shenderov, K., Huang, N.N., et al., 2012. Activation of autophagy by inflammatory signals limits IL-1beta production by targeting ubiquitinated inflammasomes for destruction. Nat. Immunol. 13, 255–263.
Song, J.X., Lu, J.H., Liu, L.F., et al., 2014. HMGB1 is involved in autophagy inhibition caused by SNCA/alpha-synuclein overexpression: a process modulated by the natural autophagy inducer corynoxine B. Autophagy 10, 144–154.
Su, Z., Wang, T., Zhu, H., et al., 2015. HMGB1 modulates Lewis cell autophagy and promotes cell survival via RAGE-HMGB1-Erk1/2 positive feedback during nutrient depletion. Immunobiology. 220, 539–544.
Sun, X., Tang, D., 2014. HMGB1-dependent and -independent autophagy. Autophagy 10, 1873–1876.
Tang, D., Kang, R., Cheh, C.W., et al., 2010a. HMGB1 release and redox regulates autophagy and apoptosis in cancer cells. Oncogene 29, 5299–5310.
Tang, D., Kang, R., Livesey, K.M., 2010b. Endogenous HMGB1 regulates autophagy. J. Cell Biol. 190, 881–892.
Tang, D., Kang, R., Livesey, K.M., et al., 2011a. High-mobility group box 1 is essential for mitochondrial quality control. Cell Metab. 13, 701–711.
Tang, D., Kang, R., Livesey, K.M., et al., 2011b. High mobility group box 1 (HMGB1) activates an autophagic response to oxidative stress. Antioxid. Redox Signal. 15, 2185–2195.
Tang, D., Kang, R., Coyne, C.B., et al., 2012. PAMPs and DAMPs: signal 0s that spur autophagy and immunity. Immunol Rev. 249, 158–175.
Tang, D., Kang, R., Van Houten, B., et al., 2014. High mobility group box 1 (HMGB1) phenotypic role revealed with stress. Mol. Med. 20, 359–362.
Tasdemir, E., Maiuri, M.C., Galluzzi, L., et al., 2008. Regulation of autophagy by cytoplasmic p53. Nat. Cell Biol. 10, 676–687.
Xie, Y., Kang, R., Sun, X., et al., 2015. Posttranslational modification of autophagy-related proteins in macroautophagy. Autophagy 11, 28–45.
Yamakuchi, M., Ferlito, M., Lowenstein, C.J., 2008. miR-34a repression of SIRT1 regulates apoptosis. Proc. Natl. Acad. Sci. U.S.A. 105, 13421–13426.
Yanai, H., Matsuda, A., An, J., et al., 2013. Conditional ablation of HMGB1 in mice reveals its protective function against endotoxemia and bacterial infection. Proc. Natl. Acad. Sci. U.S.A. 110, 20699–20704.
Yang, H., Hreggvidsdottir, H.S., Palmblad, K., et al., 2010. A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release. Proc. Natl. Acad. Sci. U.S.A. 107, 11942–11947.
Yang, L., Yu, Y., Kang, R., et al., 2012. Up-regulated autophagy by endogenous high mobility group box-1 promotes chemoresistance in leukemia cells. Leuk. Lymphoma 53, 315–322.
Yang, M., Liu, L., Xie, M., et al., 2015. Poly-ADP-ribosylation of HMGB1 regulates TNFSF10/TRAIL resistance through autophagy. Autophagy, 11.
Yang, Z., Li, L., Chen, L., et al., 2014. PARP-1 mediates LPS-induced HMGB1 release by macrophages through regulation of HMGB1 acetylation. J. Immunol. 193, 6114–6123.
Zhang, Q., Kang, R., Zeh III, H.J., et al., 2013. DAMPs and autophagy: cellular adaptation to injury and unscheduled cell death. Autophagy 9, 451–458.

Zhang, Y., Cheng, Y., Ren, X., et al., 2012. NAC1 modulates sensitivity of ovarian cancer cells to cisplatin by altering the HMGB1-mediated autophagic response. Oncogene 31, 1055–1064.
Zhao, M., Yang, M., Yang, L., et al., 2011. HMGB1 regulates autophagy through increasing transcriptional activities of JNK and ERK in human myeloid leukemia cells. BMB Rep. 44, 601–606.
Zhou, R., Yazdi, A.S., Menu, P., et al., 2011. A role for mitochondria in NLRP3 inflammasome activation. Nature 469, 221–225.
Zhu, X., Messer, J.S., Wang, Y., et al., 2015. Cytosolic HMGB1 controls the cellular autophagy/apoptosis checkpoint during inflammation. J. Clin. Invest. 125, 1098–1110.
Zong, M., Jorholt, J., Winter, J., et al., 2014. A8.24 autophagy may contribute to glucocorticoid resistance in myositis patients by maintaining muscle T cells homeostasis. Ann. Rheum. Dis. 73 (Suppl. 1), A85–A86.

CHAPTER

10

Autophagy Defects and Lafora Disease

Pankaj Kumar Singh and Subramaniam Ganesh

OUTLINE

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Abstract

Lafora disease (LD) is an adolescence-onset, genetic, and fatal form of neurodegenerative disorder with disease-defining symptoms such as progressive myoclonus epilepsy, ataxia, muscle wasting, and intellectual disabilities. One of the pathological hallmarks of the LD is the presence of Lafora bodies—inclusions of an abnormal glycogen—in the affected tissues including the neurons. LD can be caused by mutations in any one of the two known genes; the *EPM2A* gene coding for the laforin protein phosphatase and the *NHLRC1* gene coding for the malin E3 ubiquitin ligase. Laforin and malin were shown to function as a complex in several cellular processes, one of them being the autophagic pathway. Studies on animal models of the LD have demonstrated autophagic defects very early in the disease process. Autophagic defects seemingly precede any other known molecular and structural perturbations in the LD animal models, suggesting regulatory roles for the LD proteins in the disease process. At present the molecular basis of autophagy defect in LD is not clearly understood. Both mTOR-dependent and mTOR-independent mechanisms have been proposed, and a causal role for serum/glucocorticoid-induced kinase-1 in glycogen accumulation and the autophagic defect has also been shown. Here we summarize our current understanding on the role LD proteins in proteolytic pathways and their relevance to the LD pathology.

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INTRODUCTION

Lafora's progressive myoclonus epilepsy or Lafora's disease (LD; OMIM 254780) is a rare, autosomal recessive, adolescence-onset, and fatal form of progressive myoclonus epilepsy (Serratosa et al., 2012). With an age-at-onset around 15 years, the patients invariably die in about 10–15 years, most often due to respiratory failure (Ganesh et al., 2006; Singh and Ganesh, 2009; Serratosa et al., 2012). The symptoms of LD include epileptic seizures (stimulus-sensitive, grand mal, tonic–clonic, absence, visual, and myoclonic seizures), ataxia, psychosis, dementia, dysarthria, amaurosis, mutism, and muscle wasting. Being an autosomal recessive disorder, LD is more common in populations practicing consanguineous marriages. The prevalent geographic regions for LD include Spain, France and Italy, northern Africa, central and south Asia, and the Middle East (Singh and Ganesh, 2009). The defining pathology of LD is the accumulation of abnormal glycogen as Lafora bodies in affected tissues, including neurons, heart, muscle, and liver (Ganesh et al., 2006; Serratosa et al., 2012).

LD shows locus heterogeneity, resulting from mutations in the one of the two major genes known so far (Singh and Ganesh, 2009; Serratosa et al., 2012). The *EPM2A* gene, identified in the year 1998, consists of four exons and encodes a dual-specificity phosphatase, named laforin (Minassian et al., 1998; Serratosa et al., 1999; Ganesh et al., 2000). The *EPM2A* gene is evolutionarily conserved among vertebrates (Ganesh et al., 2001) and its functional orthologs have recently been identified in a few invertebrates and protists (Gentry and Pace, 2009). Laforin harbors a carbohydrate-binding domain (CBD) at its amino-terminal and a dual-specificity phosphatase domain (DSPD) at its carboxyl-terminal (Minassian et al., 2000; Ganesh et al., 2004). Through its amino-terminal CBD, laforin binds with complex carbohydrates like glycogen and polyglucosan (Ganesh et al., 2004; Chan et al., 2004a). Laforin is a unique phosphatase as it dephosphorylates both proteins (Ganesh et al., 2003; Puri et al., 2009) and complex carbohydrates (Worby et al., 2006; Tagliabracci et al., 2007). The second LD gene, *NHLRC1*, identified in the year 2003, codes for a RING finger E3 ubiquitin ligase named malin (Chan et al., 2003; Gentry et al., 2005). Laforin and malin physically interact with each other and laforin is also a substrate for malin (Gentry et al., 2005; Lohi et al., 2005). This observation led to a suggestion, and further discoveries, that laforin and malin work together as a functional complex in diverse physiological pathways (Ganesh et al., 2006). More than 100 unique mutations have been found in each gene in LD families, and all are thought to be loss-of-function mutations (Singh and Ganesh, 2009). Intriguingly, in a number of LD families neither the *EPM2A* nor the *NHLRC1* gene had any mutation, suggesting the involvement of a third locus for LD (Chan et al., 2004b; Singh and Ganesh, 2009). A strong support for this notion came from a recent study wherein a missense mutation the *PRDM8* gene was found to co-segregate in a family with an atypical form of LD (Turnbull et al., 2012). This mutation however was in heterozygous state, the phenotype thus appears to be dominant form, and the effect of this mutation on the PRDM8 protein function and in the LD phenotype is yet to be fully established.

Several studies have looked at genotype–phenotype correlations in LD. There was no significant difference in the clinical symptoms of LD patients with the *EPM2A* mutations as compared to those with *NHLRC1* mutations although the latter group of patients seemed to live longer (Gomez-abad et al., 2005; Singh et al., 2006). Such "slower" disease progression was also reported for the *EPM2A* patients (Jara-Prado et al., 2014), suggesting that

the clinical spectrum could possibly be influenced by factors other than the two LD genes (Singh and Ganesh, 2012). Indeed, a genetic modifier for LD has been reported; variation in the *PPP1R3C* gene coding for the protein targeting to glycogen was found to associate with a milder course of LD (Guerrero et al., 2011). One of the intriguing aspects of the *EPM2A* gene mutations and their effect on the laforin protein is the alternative splicing. The *EPM2A* gene in human undergoes alternative splicing and the differentially spliced transcripts have the potential to code for laforin variants with distinct amino- or carboxyl terminals (Dubey et al., 2012). The major isoform of laforin—the 331 amino acid long evolutionarily conserved laforin with CBD and DSPD (Ganesh et al., 2000)—has been studied extensively and screened for mutations, while the other variants have not yet been characterized up to the same extent. Studies have shown that the *EPM2A* gene mutations could have differential effect on distinct isoforms, and such effects could perhaps correlate with the phenotypic subgroups in LD (Dubey and Ganesh, 2008; Dubey et al., 2012).

Given the complexity in the genetics of LD and its clinical manifestations—ranging from metabolic defects to psychosis to muscle atrophy—it is not surprising to expect that the laforin and malin might play critical roles in diverse physiological processes. During the last one decade, functional analyses on cellular and animal models of LD have uncovered critical roles for laforin and malin in glucose/glycogen metabolism (Tagliabracci et al., 2007, 2008, 2011; Singh et al., 2012a), heat shock and endoplasmic reticulum (ER) stress response pathways (Vernia et al., 2009; Sengupta et al., 2011), ubiquitin-proteasomal pathway (Mittal et al., 2007; Garyali et al., 2009; Vernia et al., 2009; Rao et al., 2010), autophagy (Aguado et al., 2010; Puri et al., 2012; Criado et al., 2012; Garyali et al., 2014), *wnt* signaling pathway (Wang et al., 2006; Sharma et al., 2012), and recently in mRNA decay pathway (Singh et al., 2012b). Among these, roles for LD proteins in the glycogen metabolic pathway and the protein quality control pathway—namely, the ubiquitin–proteasome system and the autophagy—are well studied. In the subsequent sections, we review our current understanding on the role of LD proteins in proteolytic pathways and their relevance to LD pathology.

PATHOMECHANISMS OF LAFORA DISEASE

Defective Glycogen Metabolism

Lafora bodies—the pathognomonic periodic acid–Schiff-positive (PAS+) intracellular inclusion bodies in Lafora disease (LD)—are majorly composed of glucose (~90%) with a small amount of proteins (~6%) (Cavanagh, 1999). These inclusions—known as Lafora bodies—are positive for ubiquitin and advanced glycation end products (Cavanagh, 1999; Ganesh et al., 2002). Biochemical and structural studies reveal that Lafora bodies are a variant of glycogen with fewer branches and more phosphate content as compared to the later (Tagliabracci et al., 2007). Lafora body–like carbohydrate-rich inclusions are also found in Andersen's disease and in adult polyglucosan body disease, and arise primarily due to defect in the glycogen-branching enzyme (GBE), a protein involved in glycogen metabolism (Cavanagh, 1999). However, neither GBE nor any other glycogenic proteins show altered activity/level in animal models of LD (Zimmerman and Gold, 1982; Tagliabracci et al., 2008;

Turnbull et al., 2010; DePaoli-Roach et al., 2010). However, the formation of Lafora bodies and the epileptic phenotype in LD models can still be suppressed by inhibiting glycogen synthesis by knockout of the gene coding for the glycogen synthase or the protein targeting to glycogen—the regulatory subunit of protein phosphatase 1 (Turnbull et al., 2011, 2014; Pederson et al., 2013). In absence of an enzymatic defect in proteins of glycogen metabolism, excess availability of intracellular glucose, resulting upon loss of laforin or malin, seems to be the possible trigger for abnormal glycogen accumulation in LD (Singh et al., 2012b).

Lafora bodies are similar to glycogen but with an increased phosphate content than the latter (Tagliabracci et al., 2007). Recent studies suggest that the increased phosphate content could be due to the phosphomonoester formed at C2 and C3 glucosyl reside of glycogen, possibly happening due to a catalytic error made by glycogen synthase during glycogen synthesis (Tagliabracci et al., 2011), and that laforin is the enzyme that fixes this error by dephosphorylating the glycogen (Tagliabracci et al., 2007). Thus, loss of laforin would result hyperphosphorylated, aggregate-prone glycogen. A recent study proposes that the glucosyl carbon C6, and not C2/C3 as proposed earlier, is the major phosphate-harboring site in glycogen and that the glycogen synthase activity does not underlie this phosphorylation (Nitschke et al., 2013). Thus, the origin of hyperphosphorylated glycogen and its pathogenic significance in LD are yet to be unequivocally elucidated.

Defective Protein Quality Control Mechanisms

A role for laforin and malin in protein quality control system was first proposed by Mittal et al. (2007), based on their finding that proteasomal blockade results in the recruitment of laforin and malin to aggresome—a perinuclear cytoprotective structure formed to deal with the increasing demand for clearance of misfolded/aggregated proteins. Consistent with this notion, it was later shown that malin and laforin, as a functional complex, promote degradation of diverse set of cytotoxic misfolded proteins via the ubiquitin-proteasomal system (Garyali et al., 2009). A reduction in the proteolytic activity of the proteasome has also been noted in laforin- or malin-deficient cells (Vernia et al., 2009; Rao et al., 2010; Garyali et al., 2014). The laforin–malin complex was also shown to play a critical role in the heat shock–induced activation of HSF1 and in the heat shock response pathway (Sengupta et al., 2011). Laforin and malin are localized to the ER (Ganesh et al., 2000; Mittal et al., 2007), therefore a role for these proteins in ER stress response has been proposed. Indeed, laforin-deficient mice showed increased levels of ER stress markers (Bip/Grp78, CHOP, SOD2), and laforin-deficient cells showed increased apoptosis upon during ER stress (Vernia et al., 2009). Intriguingly, neuronal Lafora bodies stain positive for ubiquitin, proteasomal subunits, the ER chaperone GRP78/Bip, and molecular chaperones Hsp70/Hsc70 (Rao et al., 2010; Puri et al., 2012). Taken together, these studies suggest LD proteins to be integral components of protein quality control mechanisms and that defects in these process might lead to epilepsy and neurodegeneration in LD.

Autophagy Defects

Autophagy plays an important role in the cellular homeostasis during early development, adult hood, and in the aging process. For example, "pro-survival" role of the

autophagy has been shown during early development in *Drosophila* (Juhasz et al., 2003), *Caenorhabditis elegans* (Melendez et al., 2003), and mice (Yue et al., 2003). Its role in protecting cells against various stresses, clearance of intracellular microbes, misfolded proteins, and the damaged organelles have been shown in numerous studies (Levine and Klionsky, 2004). Autophagy thus operates at a lower level in all the cells to prevent accumulation of damaged or aberrant cellular components and performs cellular quality control. Impediments in the autophagy underlie the basis of several neurodegenerative disorders like Alzheimer's disease, Parkinson's disease, Huntington disease, and amyotrophic lateral sclerosis (Nixon, 2013) and is now gaining considerable attention in LD as well (see below).

The process of autophagy is defective in LD mouse models and in the fibroblast derived from LD patients (Aguado et al., 2010; Criado et al., 2012; Puri et al., 2012). The primary defect identified is at the level of reduced autophagosome synthesis. Defective autophagy is seen in as early as 16-day-old malin or laforin-deficient mice (Criado et al., 2012)—a stage at which the animal is asymptomatic and without visible Lafora bodies in the neurons. Thus, defective autophagy could possibly contribute to Lafora body formation and neurodegeneration in LD (Puri and Ganesh, 2010). Lafora bodies may also contribute to autophagy defects; studies in LD mice models have revealed that several critical players of autophagy and endosomal–lysosomal trafficking (proteins like p62, Rab5, and Rab7) are sequestered to Lafora bodies (Puri et al., 2012). These inclusions thus could perturb the endosomal–lysosomal trafficking and lysosomal function and could possibly explain progressive nature of the disease (Puri et al., 2012). Intriguingly, Lafora bodies do not seem to colocalize with autophagosome and/or lysosome in laforin-deficient mice, suggesting that Lafora bodies are not cleared by autophagy (Puri et al., 2012). Thus defective autophagic process appears to be both causative and consequential events in LD brains (Puri and Ganesh, 2010, 2012).

Autophagy is a complex process and is regulated by several external and internal stimuli and numerous signaling pathways (He and Klionsky, 2009). The mammalian target of rapamycin (mTORC1) is a central growth regulator kinase that, in response to nutritional status, growth factor, and other signals, negatively regulates the autophagic process (Jung et al., 2010). Intriguingly, mTORC1 is hyperactive in laforin-deficient mice, thereby elucidating the molecular basis of autophagy defect in LD (Aguado et al., 2010). Conversely, overexpression of laforin suppressed mTORC1 activity and induced autophagy (Aguado et al., 2010). mTORC1 is negatively regulated by the TSC1/hamartin and TSC2/tuberin complexes (Zhang et al., 2003). TSC1 and TSC2 are proteins pathologically linked to tuberous sclerosis complex (TSC), a genetic disorder characterized by seizures, mental retardation, and renal dysfunction (Aicher et al., 2001; Tee et al., 2003; Inoki et al., 2005). Intriguingly, laforin overexpression failed to increase autophagosome numbers in TSC2-deficient cells suggesting laforin may require TSC2 for its control over mTORC1 (Aguado et al., 2010). The activity of TSC2 is regulated by PI3K/AKT-dependent phosphorylation on its residue Thr-1462 (Huang and Manning, 2008). However, Thr-1462 did not show any difference in the phosphorylation level in laforin-deficient mice (Depaoli-Roach et al., 2012), suggesting TSC2 may not play a role in the laforin-mediated autophagy regulation. Malin-deficient mice also showed similar autophagy defect but with no change in the mTORC1 activity, suggesting an mTORC1-independent regulation of autophagy in these mice (Criado et al., 2012).

A recent study elucidated a functional link between laforin and mTOR and proposed an attractive model to explain the increased mTORC1 activity and autophagy defect in

laforin-deficient tissues (Singh et al., 2013). As per this model, the mTORC1 activation in laforin-deficient condition is glucose-dependent since depletion of glucose from culture media prevented the mTORC1 activation in laforin-deficient fibroblast (Singh et al., 2013). mTOR is a multi-protein functional complex whose activity is sensitive to nutritional status of the cell. For example, nutrient deprivation inhibits mTORC1 complex by stabilizing the interaction between mTOR and its adaptor protein raptor (Kim et al., 2002). Alternatively, glucose and glycolytic flux activates mTORC1 complex by enhancing its association with the Rheb GTPase (Lee et al., 2009). Since loss of laforin is known to enhance cellular glucose uptake, it is reasonable to assume that increased glucose level might activate mTORC1 in laforin-deficient state (Singh et al., 2013). Laforin deficiency also triggers the activation of serum/glucocorticoid-induced kinase-1 (SGK1) in an mTORC1-dependent manner (Singh et al., 2013). SGK1 is a downstream component of the PI3K signaling pathway that regulates membrane localization of several ion channels and transporter proteins (Lang et al., 2006). SGK1 is known to be phosphorylated and activated by mTORC1 in a cell-type specific manner (Hong et al., 2008). Conforming to this model, mTORC1 inhibition prevented the SGK1 activation in laforin-deficient cells (Singh et al., 2013). SGK1 inhibition reduced glucose uptake and also prevented the activation of mTORC1 in laforin-deficient cells, supporting the hypothesis that mTOR1 activation is glucose-dependent (Singh et al., 2013) in these cells. Thus, the existence of a feed-forward regulatory mechanism between mTOR and SGK1 under laforin-deficient condition was proposed (Singh et al., 2013).

CONCLUSIONS

In the absence of an obvious defect in glycogen metabolism and the emerging knowledge that metabolites like glycogen and lipids are degraded via autophagy, it is reasonable to speculate that defective autophagy might contribute to the accumulation of abnormal glycogen in LD tissues. Thus, defective autophagic process is emerging as the central player in the plethora of pathologies in LD. Glycogen in the liver and muscle tissues are known to be degraded via autophagy. However whether or not it is true for the neuronal glycogen is yet to be established. Indeed, a cause–effect relationship between neuronal glycogen and autophagy is debatable, particularly in light of a recent report that inducing glycogen synthesis in the brain impairs autophagy (Duran et al., 2014). There appears to be a complex relationship between these two metabolic pathways (Singh and Singh, 2015), and therefore more work is required to understand the role of glycogen and autophagy in the neuronal dysfunctions.

References

Aguado, C., Sarkar, S., Korolchuk, V.I., et al., 2010. Laforin, the most common protein mutated in Lafora disease, regulates autophagy. Hum. Mol. Genet. 19, 2867–2876.

Aicher, L.D., Campbell, J.S., Yeung, R.S., 2001. Tuberin phosphorylation regulates its interaction with hamartin. Two proteins involved in tuberous sclerosis. J. Biol. Chem. 276, 21017–21021.

Cavanagh, J.B., 1999. Corpora-amylacea and the family of polyglucosan diseases. Brain Res. Rev. 29, 265–295.

Chan, E.M., Young, E.J., Ianzano, L., et al., 2003. Mutations in NHLRC1 cause progressive myoclonus epilepsy. Nat. Genet. 35, 125–127.

Chan, E.M., Ackerley, C.A., Lohi, H., et al., 2004a. Laforin preferentially binds the neurotoxic starch-like polyglucosans, which form in its absence in progressive myoclonus epilepsy. Hum. Mol. Genet. 13, 1117–1129.
Chan, E.M., Omer, S., Ahmed, M., et al., 2004b. Progressive myoclonus epilepsy with polyglucosans (Lafora disease): evidence for a third locus. Neurology 63, 565–567.
Criado, O., Aguado, C., Gayarre, J., et al., 2012. Lafora bodies and neurological defects in malin-deficient mice correlate with impaired autophagy. Hum. Mol. Genet. 21, 1521–1533.
DePaoli-Roach, A.A., Tagliabracci, V.S., Segvich, D.M., et al., 2010. Genetic depletion of the malin E3 ubiquitin ligase in mice leads to Lafora bodies and the accumulation of insoluble laforin. J. Biol. Chem. 285, 25372–25381.
DePaoli-Roach, A.A., Segvich, D.M., Meyer, C.M., et al., 2012. Laforin and malin knockout mice have normal glucose disposal and insulin sensitivity. Hum. Mol. Genet. 21, 1604–1610.
Dubey, D., Ganesh, S., 2008. Modulation of functional properties of laforin phosphatase by alternative splicing reveals a novel mechanism for the EPM2A gene in Lafora progressive myoclonus epilepsy. Hum. Mol. Genet. 17, 3010–3020.
Dubey, D., Parihar, R., Ganesh, S., 2012. Identification and characterization of novel splice variants of the human EPM2A gene mutated in Lafora progressive myoclonus epilepsy. Genomics 99, 36–43.
Duran, J., Gruart, A., García-Rocha, M., et al., 2014. Glycogen accumulation underlies neurodegeneration and autophagy impairment in Lafora disease. Hum. Mol. Genet. 23, 3147–3156.
Ganesh, S., Agarwala, K.L., Ueda, K., et al., 2000. Laforin, defective in the progressive myoclonus epilepsy of Lafora type, is a dual-specificity phosphatase associated with polyribosomes. Hum. Mol. Genet. 9, 2251–2261.
Ganesh, S., Agarwala, K.L., Amano, K., et al., 2001. Regional and developmental expression of Epm2a gene and its evolutionary conservation. Biochem. Biophys. Res. Commun. 283, 1046–1053.
Ganesh, S., Delgado-Escueta, A.V., Sakamoto, T., et al., 2002. Targeted disruption of the Epm2a gene causes formation of Lafora inclusion bodies, neurodegeneration, ataxia, myoclonus epilepsy and impaired behavioral response in mice. Hum. Mol. Genet. 11, 1251–1262.
Ganesh, S., Tsuritamni, N., Suzuki, T., et al., 2003. The Lafora disease gene product laforin interacts with HIRP5, a phylogenetically conserved protein containing a NifU-like domain. Hum. Mol. Genet. 12, 2359–2368.
Ganesh, S., Tsurutani, N., Suzuki, T., et al., 2004. The carbohydrate-binding domain of Lafora disease protein targets Lafora polyglucosan bodies. Biochem. Biophys. Res. Commun. 313, 1101–1109.
Ganesh, S., Puri, R., Singh, S., et al., 2006. Recent advances in the molecular basis of Lafora's progressive myoclonus epilepsy. J. Hum. Genet. 51, 1–8.
Garyali, P., Siwach, P., Singh, P.K., et al., 2009. The malin–laforin complex suppresses the cellular toxicity of misfolded proteins by promoting their degradation through the ubiquitin-proteasome system. Hum. Mol. Genet. 18, 688–700.
Garyali, P., Segvich, D.M., DePaoli-Roach, A.A., et al., 2014. Protein degradation and quality control in cells from laforin and malin knockout mice. J. Biol. Chem. 289, 20606–20614.
Gentry, M.S., Pace, R.M., 2009. Conservation of the glucan phosphatase laforin is linked to rates of molecular evolution and the glucan metabolism of the organism. BMC Evol. Biol. 9, 138.
Gentry, M.S., Worby, C.A., Dixon, J.E., 2005. Insights into Lafora disease: malin is an E3 ubiquitin ligase that ubiquitinates and promotes the degradation of laforin. Proc. Natl. Acad. Sci. U.S.A. 102, 8501–8506.
Gomez-Abad, C., Gomez-Garre, P., Gutierrez-Delicado, E., et al., 2005. Lafora disease due to EPM2B mutations: a clinical and genetic study. Neurology 64, 982–986.
Guerrero, R., Vernia, S., Sanz, R., et al., 2011. A PTG variant contributes to a milder phenotype in Lafora disease. PLoS One 6, e21294.
He, C., Klionsky, D.J., 2009. Regulation mechanisms and signaling pathways of autophagy. Annu. Rev. Genet. 43, 67–93.
Hong, F., Larrea, M.D., Doughty, C., et al., 2008. mTOR-raptor binds and activates SGK1 to regulate p27 phosphorylation. Mol. Cell 30, 701–711.
Huang, J., Manning, B.D., 2008. The TSC1–TSC2 complex: a molecular switchboard controlling cell growth. Biochem. J. 412, 179–190.
Inoki, K., Corradetti, M.N., Guan, K.L., 2005. Dysregulation of the TSC–mTOR pathway in human disease. Nat. Genet. 37, 19–24.
Jara-Prado, A., Ochoa, A., Alonso, M.E., et al., 2014. Late onset Lafora disease and novel EPM2A mutations: breaking paradigms. Epilepsy Res. 108, 1501–1510.
Juhasz, G., Csikos, G., Sinka, R., et al., 2003. The *Drosophila* homolog of Aut1 is essential for autophagy and development. FEBS Lett. 543, 154–158.

Jung, C.H., Ro, S.H., Cao, J., et al., 2010. mTOR regulation of autophagy. FEBS Lett. 584, 1287–1295.
Kim, D.H., Sarbassov, D.D., Ali, S.M., et al., 2002. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell 110, 163–175.
Lang, F., Bohmer, C., Palmada, M., et al., 2006. (Patho)physiological significance of the serum- and glucocorticoid-inducible kinase isoforms. Physiol. Rev. 86, 1151–1178.
Lee, M.N., Ha, S.H., Kim, J., et al., 2009. Glycolytic flux signals to mTOR through glyceraldehyde-3-phosphate dehydrogenase-mediated regulation of Rheb. Mol. Cell. Biol. 29, 3991–4001.
Levine, B., Klionsky, D.J., 2004. Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev. Cell 6, 463–477.
Liu, Y., Wang, Y., Wu, C., et al., 2009. Deletions and missense mutations of EPM2A exacerbate unfolded protein response and apoptosis of neuronal cells induced by endoplasm reticulum stress. Hum. Mol. Genet. 18, 2622–2631.
Lohi, H., Ianzano, L., Zhao, X.C., et al., 2005. Novel glycogen synthase kinase 3 and ubiquitination pathways in progressive myoclonus epilepsy. Hum. Mol. Genet. 14, 2727–2736.
Melendez, A., Talloczy, Z., Seaman, M., et al., 2003. Autophagy genes are essential for dauer development and life-span extension in *C. elegans*. Science 301, 1387–1391.
Minassian, B.A., Lee, J.R., Herbrick, J.A., et al., 1998. Mutations in a gene encoding a novel protein tyrosine phosphatase cause progressive myoclonus epilepsy. Nat. Genet. 20, 171–174.
Minassian, B.A., Ianzano, L., Meloche, M., et al., 2000. Mutation spectrum and predicted function of laforin in Lafora's progressive myoclonus epilepsy. Neurology 55, 341–346.
Mittal, S., Dubey, D., Yamakawa, K., et al., 2007. Lafora disease proteins malin and aforin are recruited to aggresomes in response to proteasomal impairment. Hum. Mol. Genet. 16, 753–762.
Nitschke, F., Wang, P., Schmieder, P., et al., 2013. Hyperphosphorylation of glucosyl C6 carbons and altered structure of glycogen in the neurodegenerative epilepsy Lafora disease. Cell Metab. 17, 756–767.
Nixon, R.A., 2013. The role of autophagy in neurodegenerative disease. Nat. Med. 19, 983–997.
Pederson, B.A., Turnbull, J., Epp, J.R., et al., 2013. Inhibiting glycogen synthesis prevents Lafora disease in a mouse model. Ann. Neurol. 74, 297–300.
Puri, R., Ganesh, S., 2010. Laforin in autophagy: a possible link between carbohydrate and protein in Lafora disease? Autophagy 6, 1229–1231.
Puri, R., Ganesh, S., 2012. Autophagy defects in Lafora disease: cause or consequence? Autophagy 8, 289–290.
Puri, R., Suzuki, T., Yamakawa, K., et al., 2009. Hyperphosphorylation and aggregation of Tau in laforin-deficient mice, an animal model for Lafora disease. J. Biol. Chem. 284, 22657–22663.
Puri, R., Suzuki, T., Yamakawa, K., et al., 2012. Dysfunctions in endosomal-lysosomal and autophagy pathways underlie neuropathology in a mouse model for Lafora disease. Hum. Mol. Genet. 21, 175–184.
Rao, S.N., Maity, R., Sharma, J., et al., 2010. Sequestration of chaperones and proteasome into Lafora bodies and proteasomal dysfunction induced by Lafora disease-associated mutations of malin. Hum. Mol. Genet. 19, 4726–4734.
Sengupta, S., Badhwar, I., Upadhyay, M., et al., 2011. Malin and laforin are essential components of a protein complex that protects cells from thermal stress. J. Cell. Sci. 124, 2277–2286.
Serratosa, J.M., Gomez-Garre, P., Gallardo, M.E., et al., 1999. A novel protein tyrosine phosphatase gene is mutated in progressive myoclonus epilepsy of the Lafora type (EPM2). Hum. Mol. Genet. 8, 345–352.
Serratosa, J.M., Minassian, B.A., Ganesh, S., 2012. Progressive myoclonus epilepsy of Lafora. In: Noebels, J., Avoli, M., Rogawski, M. (Eds.), Jasper's Basic Mechanisms of the Epilepsies, fourth ed. Oxford University Press, USA, pp. 874–878.
Sharma, J., Mulherkar, S., Mukherjee, D., et al., 2012. Malin regulates Wnt signaling pathway through degradation of dishevelled2. J. Biol. Chem. 287, 6830–6839.
Singh, P.K., Singh, S., 2015. Changing shapes of glycogen–autophagy nexus in neurons: perspective from a rare epilepsy. Front. Neurol. 6, 14. http://dx.doi.org/10.3389/fneur.2015.00014.
Singh, P.K., Singh, S., Ganesh, S., 2012a. The laforin–malin complex negatively regulates glycogen synthesis by modulating cellular glucose uptake via glucose transporters. Mol. Cell. Biol. 32, 652–663.
Singh, P.K., Singh, S., Ganesh, S., 2013. Activation of serum/glucocorticoid-induced kinase (SGK1) underlies increased glycogen levels, mTOR activation, and autophagy defects in Lafora disease. Mol. Biol. Cell 24, 3776–3786.

Singh, S., Ganesh, S., 2009. Lafora progressive myoclonus epilepsy: a meta-analysis of reported mutations in the first decade following the discovery of the EPM2A and NHLRC1 genes. Hum. Mutat. 30, 715–723.
Singh, S., Ganesh, S., 2012. Phenotype variations in Lafora progressive myoclonus epilepsy: possible involvement of genetic modifiers? J. Hum. Genet. 57, 283–285.
Singh, S., Sethi, I., Francheschetti, S., et al., 2006. Novel NHLRC1 mutations and genotype–phenotype correlations in patients with Lafora's progressive myoclonus epilepsy. J. Med. Genet. 43, e48.
Singh, S., Singh, P.K., Bhadauriya, P., et al., 2012b. Lafora disease E3 ubiquitin ligase malin is recruited to the processing bodies and regulates the microRNA-mediated gene silencing process via the decapping enzyme Dcp1a. RNA Biol. 9, 1440–1449.
Tagliabracci, V.S., Turnbull, J., Wang, W., et al., 2007. Laforin is a glycogen phosphatase, deficiency of which leads to elevated phosphorylation of glycogen *in vivo*. Proc. Natl. Acad. Sci. U.S.A. 104, 19262–19266.
Tagliabracci, V.S., Girard, J.M., Segvich, D., et al., 2008. Abnormal metabolism of glycogen phosphate as a cause for Lafora disease. J. Biol. Chem. 283, 33816–33825.
Tagliabracci, V.S., Heiss, C., Karthik, C., et al., 2011. Phosphate incorporation during glycogen synthesis and Lafora disease. Cell Metab. 13, 274–282.
Tee, A.R., Manning, B.D., Roux, P.P., et al., 2003. Tuberous sclerosis complex gene products, tuberin and hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. Curr. Biol. 13, 1259–1268.
Turnbull, J., Wang, P., Girard, J.M., et al., 2010. Glycogen hyperphosphorylation underlies Lafora body formation. Ann. Neurol. 68, 925–933.
Turnbull, J., Epp, J.R., Goldsmith, D., et al., 2014. PTG protein depletion rescues malin-deficient Lafora disease in mouse. Ann. Neurol. 75, 442–446.
Turnbull, J., DePaoli-Roach, A.A., Zhao, X., et al., 2011. PTG depletion removes Lafora bodies and rescues the fatal epilepsy of Lafora disease. PLoS Genet. 7, e1002037.
Turnbull, J., Girard, J.M., Lohi, H., et al., 2012. Early-onset Lafora body disease. Brain 135, 2684–2698.
Vernia, S., Rubio, T., Heredia, M., et al., 2009. Increased endoplasmic reticulum stress and decreased proteasomal function in Lafora disease models lacking the phosphatase laforin. PLoS One 4, e5907.
Wang, Y., Liu, Y., Wu, C., et al., 2006. Epm2a suppresses tumor growth in an immunocompromised host by inhibiting Wnt signaling. Cancer Cell 10, 179–190.
Worby, C.A., Gentry, M.S., Dixon, J.E., 2006. Laforin, a dual specificity phosphatase that dephosphorylates complex carbohydrates. J. Biol. Chem. 281, 30412–30418.
Yue, Z., Jin, S., Yang, C., et al., 2003. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. Proc. Natl. Acad. Sci. U.S.A. 100, 15077–15082.
Zhang, H., Cicchetti, G., Onda, H., et al., 2003. Loss of Tsc1/Tsc2 activates mTOR and disrupts PI3K-Akt signaling through downregulation of PDGFR. J. Clin. Invest. 2112, 1223–1233.
Zimmerman, C.P., Gold, A.M., 1982. Glycogen branching enzyme in Lafora myoclonus epilepsy. Biochem. Med. 28, 83–93.

CHAPTER

11

Regulation of Autophagy in Parkinson's Disease: Insights into New Therapeutic Targets

Ana Raquel Esteves, Diana Filipa Silva, Maria Gonçalves-Fernandes, Rui Gomes and Sandra Morais Cardoso

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Abstract

Neurons, long-lived postmitotic cells, are highly dependent on quality control mechanisms to maintain a healthy intracellular environment. A continuous evaluation of protein and organelle "quality" allows a coordinated action between repair and clearance of damage proteins and dysfunctional organelles. A great body of evidence links mitochondrial and lysosomal dysfunction, and protein misfolding to Parkinson's disease (PD) etiopathogenesis. Although the mechanism underlying protein accumulation remains unclear, it is believed that impaired autophagy can trigger downstream pathogenic events, leading to neurodegeneration of different subset of neurons. Indeed, it has been described that age-dependent impairments in the intracellular clearance mechanisms result in the progressive accumulation of damaged organelles and aggregates of aberrant proteins. In view of this, we review the latest advances on autophagy involvement in PD etiopathogenesis and discuss potential disease-modifying strategies.

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INTRODUCTION

Autophagy was first denominated by Christian de Duve and comes from Greek, meaning self-eating. This term, related to the degradation of intracellular components in the lysosomal system, includes three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA), which differ in the mechanism by which materials are delivered to the lysosomes. In microautophagy, the cytosolic elements to be degraded are engulfed by the lysosome itself that deforms creating an invagination containing the substrates. CMA degrades soluble cytosolic proteins which bind to a receptor at the lysosomal membrane and mediates their translocation to the lysosomal lumen. Macroautophagy, referred to hereafter as autophagy, is a degradative pathway that allows the removal of cellular organelles, such as mitochondria, and long-lived proteins, involving the generation of autophagosomes, double-membrane vesicles that enclose a portion of the cytosol and target cytoplasmic components to the lysosome. Damaged mitochondria are eliminated through a selective autophagic mechanism denominated mitophagy which insures the maintenance of a healthy mitochondrial pool. In fact, autophagy was first described as a mainly starvation-inducible process, leading to bulk degradation of substrates but now it is recognized its constitutive role as a quality control mechanism. This cellular housekeeping function includes the removal of damaged organelles, pathogens, and inclusions which implicates autophagy dysfunction in a broad number of disorders: cancer, heart and neurodegenerative diseases, inflammation, and infection. The selectivity of autophagy is accomplished by adaptor proteins, known as autophagic receptors that promote selective degradation. These adaptors comprise proteins such as p62/SQSTM1 (Sequestosome 1), implicated in the selective elimination of ubiquitinated proteins, various organelles such as mitochondria and pathogens. Autophagy is a well-orchestrated process involving several steps. During starvation the inhibition of mTOR complex activates autophagy. Otherwise, macroautophagy can be induced independently of mTOR, namely, through the alteration of transcription of autophagic genes or reduced levels of inositol 1,4,5-trisphosphate (IP3) (Sarkar et al., 2009). In mammals, the induction is controlled by Yeast homologs of autophagy-related genes (*ATGs*), the ULK1/2-ATG13-FIP200 complex (Rogov et al., 2014). Then nucleation proceeds with the formation of a large protein complex, Beclin 1/Class III phosphatidylinositol-3-kinase (PI3K), which recruits Atg proteins to the membrane of the preautophagosomes to promote elongation and expansion of the autophagosome. This enables the conjugation of phosphatidylethanolamine (PE) to the microtubule-associated protein 1 light chain 3 (LC3)-I to form LC3-II, leading to the translocation of LC3 from cytoplasm to the membrane of the preautophagosomes. After the elongation step, autophagosomes fuse with several types of vesicles from the endosomal/lysosomal pathways which allow autophagosome maturation. The fusion with lysosomes that contain hydrolases responsible for material degradation requires the action of Rab7 and lysosomal membrane protein LAMP-2 (Wong et al., 2011).

In the case of neurons, highly differentiated cells with widespread synapses, branched dendrites, and axons that can achieve a considerable size, the autophagic process is distinguishable within cell compartments (Damme et al., 2014). Recently it was shown that autophagosomes are preferentially formed in distal ends of axons of primary neurons allowing the renewal of dysfunctional mitochondria and long-lived proteins, necessary at

the synapse (Maday and Holzbaur, 2014). The functional compartmentalization of neuronal cells requires microtubule network for the formation and maturation of autophagosomes containing degrading materials. The autophagosomes are transported by retrograde transport along microtubules to the cell soma, where most of the lysosomes are located.

Because neurons are postmitotic cells, the inability of diffusing toxic materials through cell division makes neurons especially sensitive to misfolded proteins and dysfunctional organelles accumulation and places autophagy as a crucial process in these cells (Maday and Holzbaur, 2014). Despite autophagosomes are scarce in neurons, which may be due to high autophagic efficiency (Furuta et al., 2010), the ablation of autophagy-required genes in neurons promotes axonal degeneration and cell death (Hara et al., 2006).

The stage that Parkinson's disease (PD) research has reached undoubtedly shows that PD is not a brain-limited disease, instead many pathological hallmarks have been found in peripheral tissues. Autophagic dysfunction in postmitotic neurons jeopardizes brain function, while in peripheral tissues it can be postpone due to cell division. This fact raises the challenge on understanding the mechanisms of PD pathology and their regulation in order for new therapeutic strategies can be applied.

AUTOPHAGY IN PD

PD is a well-known neurodegenerative disease characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), and by the intracellular accumulation of α-synuclein (ASYN)-containing Lewy bodies (LBs), in surviving SNpc neurons.

Although the multifactorial causes of PD are still a matter under debate nowadays, all the pathogenesis studies converge on mitochondrial dysfunction, oxidative stress, and consequent protein aggregation, which are strictly correlated with autophagy. Autophagy is a key player in cell homeostasis, and is essential for the bulk degradation of cytoplasmic content, especially under a stressful environment. Therefore, an efficient autophagy is critical for the proper functioning of cells, particularly in neurons and *other long-lived cells*, of postmitotic nature and extreme size and polarization, where the accumulation of aggregated proteins or damaged organelles may compromise their survival.

The Role of Autophagy in PD

One of first evidences for autophagy in PD came from the discovery that ASYN in its native form is a substrate of the CMA, and, interestingly, the CMA activity was found to be decreased in *postmortem* SN of PD brains (Alvarez-Erviti et al., 2010). Unlike wild-type ASYN, its mutant counterparts were reported to block the CMA (Cuervo et al., 2004; Martinez-Vicente et al., 2008) and gain toxic function in the cytoplasm (Cuervo et al., 2004), suggesting that CMA is crucial to limit the ASYN oligomerization, and consequent toxicity (Nixon, 2013). Posttranslational modified ASYN also promote CMA blockage, making unfeasible their subsequent degradation by this pathway (Martinez-Vicente et al., 2008). In addition to ASYN, an impairment of CMA may also trigger neurodegeneration via an ineffective degradation of other proteins (Banerjee et al., 2010), as, for example, the MEF2D,

which is a transcription factor essential for neuronal survival, and whose levels are elevated in *postmortem* PD brains (Yang et al., 2009).

Macroautophagy is then activated as a compensatory route to remove the ASYN aggregates (Massey et al., 2006), and its oligomeric intermediates (Lee et al., 2004), considered to be the most toxic forms of ASYN (Outeiro et al., 2008). Indeed, it was identified an abnormal presence of autophagosomes in dopaminergic SNpc neurons of *postmortem* PD patients. In agreement, Dehay et al. (2010) also observed an accumulation of autophagic structures in *postmortem* PD nigra samples, where LBs were strongly marked with the autophagy marker LC3, which suggests an autophagy induction as an attempt to ameliorate ASYN pathology (Cook et al., 2012). Additionally, they also reported a decrease in the lysosomal marker LAMP-1 (Dehay et al., 2010). Similar evidences were found in sPD cytoplasmic hybrids (cybrids) (Arduino et al., 2012, 2013; Esteves et al., 2010, 2014a), a cell model developed, in which the original mitochondrial DNA (mtDNA) is replaced by PD patient mtDNA; and in differentiated sPD cybrids, Rho0 cells, primary cortical neurons with damaged mitochondria, and in PD patient lymphocytes (Arduino et al., 2013). Finally, studies using different animal models also exhibit autophagosomes accumulation (Crews et al., 2010; Dehay et al., 2010).

Further support of macroautophagy as a key player in PD pathogenesis came from studies where brain-specific deletion of the autophagy-related gene *Atg7* in the mice dopaminergic neurons of the SNpc leads to a phenotype closely similar to what is observed in PD (Fecto et al., 2014). In addition, other studies demonstrated that macroautophagy inhibition potentiates ASYN aggregation and toxicity in a cellular and mouse models (Crews et al., 2010), as well as an induction with different types of compounds results in an improvement of the molecular traits of PD (Crews et al., 2010; Ghavami et al., 2014). Furthermore, overexpression of ASYN promotes the formation of inclusions in the cytosol, thought to be abnormal autophagic vesicles (Spencer et al., 2009). Following overexpression of Beclin 1, which increases autophagy, a reduction of accumulated ASYN and consequent neurotoxicity was observed, thus confirming that autophagy is upregulated to ameliorate ASYN toxicity and to maintain neuronal functionality (Spencer et al., 2009).

A number of current studies have placed mitochondria dysfunction as a mechanism of PD pathology (Cardoso, 2011). Consistent with this, mitochondria has been found within autophagosomes (Takano-Ohmuro et al., 2000), being thus a substrate of autophagy—mitophagy (Narendra et al., 2009). In fact, the loss of autophagy-related proteins, Atg5 and Atg7, results in damaged mitochondria accumulation (Mortensen et al., 2010).

Aging is a risk factor for the development of sporadic PD, and aged organisms have a decrease in CMA and macroautophagy activity, as well as an accumulation of impaired mitochondria (Fecto et al., 2014).

As final evidence, the familial PD proteins PINK1 (PTEN-induced kinase 1), parkin, DJ-1, and Leucine-rich repeat kinase 2 (LRRK2) have been linked to the autophagic pathway. PINK1, a voltage-sensitive kinase, accumulates on the outer mitochondrial membrane of damaged mitochondria (Narendra et al., 2010). Such accumulation of PINK1 facilitates the recruitment of Parkin to mitochondria, for the ubiquitylation of outer mitochondrial proteins, which are thought to enable the clearance of damaged mitochondria (Okatsu et al., 2015). Accordingly, mutations in PINK1 or Parkin seen in familial PD disrupt such translocation, thereby interfering with the removal of impaired mitochondria (Menzies et al., 2011).

A depletion of DJ-1 leads to mitochondrial dysfunction and impaired autophagy (Thomas et al., 2011). And, finally, LRRK2, the most common genetic cause of PD, interacts and increases ASYN levels (Carballo-Carbajal et al., 2010), and significantly alters autophagosome formation (Ebrahimi-Fakhari et al., 2012).

Collectively, these observations highlight the role of all autophagy mechanisms in pathogenesis of PD and shed light for the development of new disease-modifying treatments through autophagy manipulation.

Mitochondria Dependency

The regulation of autophagy by mTOR is dependent on amino acid and ATP levels, which result from starvation. Although some controversy exists on literature, it is hypothesized that AMPK (AMP-activated protein kinase) starts autophagy through an indirect mechanism, inhibiting mTOR activity (Mao and Klionsky, 2011). Low ATP levels activate AMP-dependent protein kinase (AMPK) pathway and stimulate autophagy (Altman and Rathmell, 2012). AMPK is an evolutionary conserved cellular energy controller, a checkpoint of nutrient sensing and energy status (Mao and Klionsky, 2011). In fibroblasts from patients carrying inherited complex I deficiency, it is triggered an adaptatory response with the increase of AMPK protein levels accompanied by a stimulation of mitochondrial biogenesis and rescue of mitochondrial membrane potential (Distelmaier et al., 2015). Mitochondrial dysfunction comes in hand with enhanced reactive oxygen species (ROS) formation. The resultant oxidative stress primarily triggers mitochondria itself and the elimination of damaged mitochondria through mitophagy requires the activation of AMPK (Wu et al., 2014). The role of AMPK in eliminating dysfunctional mitochondria was further supported by the observation that in AMPK knockout mice there is the accumulation of p62 along with ubiquitylated proteins and dysfunctional mitochondria in the liver of these animals (Wu et al., 2014). The elimination of mitochondria through mitophagy is regulated through a mechanism elegantly orchestrated. Mutations in PINK1 and Parkin give rise to early-onset PD, both clinically very similar, suggesting that these proteins mediate the same signaling pathway (Narendra et al., 2012). PINK1 is a mitochondrial protein and in nonpathogenic conditions is rapidly cleaved and degraded. In a situation of mitochondrial dysfunction, the loss of mitochondrial membrane potential ($\Delta\psi_{mit}$) triggers the accumulation of PINK1 and Parkin, an E3 ubiquitin ligase, is recruited from the cytosol (Kondapalli et al., 2012). It is hypothesized that, in these conditions of mitochondrial depolarization, PINK1 is relocated from the inner to the outer mitochondrial membrane and is no longer accessible for presenilin-associated rhomboid-like (PARL) (Jin et al., 2010). To test this theory cells were treated with carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), a mitochondrial uncoupler, and PINK1 becomes rapidly stabilized and phosphorylates Parkin Ser^{65} (Kondapalli et al., 2012). The elimination of ubiquitylated mitochondria is perpetrated by histone deacetylase 6 (HDAC6) and p62 which recognize the ubiquitin signals and assemble autophagy machinery to encapsulate dysfunctional mitochondria in autophagosomes for degradation (Lee et al., 2010b). In mononuclear cells from fibromyalgia patients, the loss of $\Delta\psi_{mit}$ is accompanied by increments in mitophagy, where mitochondria are found in superior numbers within autophagosomes (Cordero et al., 2010). In cybrid cell lines, containing sporadic PD subject's mtDNA, and show inherent mitochondrial dysfunction, PINK1 accumulates on

mitochondria and activates Parkin (Silva et al., unpublished data). Although the initial steps of autophagy are occurring, with engulfing of dysfunctional mitochondria by autophagosomes, proper elimination by fusion with lysosomes is impeded and dysfunctional mitochondria accumulate (Arduino et al., 2012).

Mitochondrial dynamics is essential for the maintenance of a healthy pool of mitochondria due to the segregation of damaged mitochondria for degradation. A number of observations have postulated a close relation between mitochondrial dynamics and mitophagy, where changes in expression of fusion and fission proteins modify autophagy. Mitochondrial fission, through the control of DRP1 (Dynamin-related protein 1) and Fis 1 (Fission 1), is a crucial step for mitophagy since inhibition of mitochondrial fission causes mitophagy rates to decrease (Kubli and Gustafsson, 2012). In various disease models mitochondrial fragmentation due to increased fission has been characterized as a stress-mediated response, in an attempt to eliminate the dysfunctional parts of mitochondria. In a PD model of PINK1-deficient cells, mitochondrial interconnectivity and size show a fragmented mitochondrial network (Dagda et al., 2009). In sporadic PD cybrids, it was found that fragmentation of mitochondrial network is linked to increased DRP1 expression and Opa 1 (Optic atrophy 1) long isoform cleavage (Santos et al., 2014), and the knockdown of DRP1 showed to be protective in these conditions.

Microtubule Dependency

The first connection between autophagy and microtubule network was proposed for more than a decade ago. This study reported that use of vincristine or vinblastine, depolarizing agents of microtubules, inhibits a mechanism of protein degradation involving cytoplasmic autophagy in rat embryo fibroblasts. More recent studies confirmed that the use of these agents in rat hepatocytes and kidney epithelial cells causes the disruption of the microtubule network interfering with fusion of autophagosomes with lysosomes. However, other studies show that disruption of microtubules only leads to a delay in autophagy, not completely blocking this process in mammalian cells (Jahreiss et al., 2008). It was demonstrated a key role for microtubules in autophagic process namely in the formation of autophagosomes and motility of these. It was also found that motor proteins associated with microtubules had an important role in the transport of autophagosomes along microtubules toward lysosomes. The inhibition of dynein function leads to a decrease in autophagy-mediated clearance of ASYN (Jahreiss et al., 2008).

Relevant to PD pathology, it has been reported that ASYN, LRRK2, PINK1, or Parkin mutants alter the intracellular traffic (Schon and Przedborski, 2011). The impaired axonal transport was correlated with ASYN aggregates in sporadic PD brains (Chu et al., 2012). The interaction of ASYN with microtubules was shown *in vitro* and *in vivo*, using overexpression and mutant forms of ASYN which causes disruption of microtubule network dependent trafficking (Zhou et al., 2010). Other studies using oligomeric ASYN forms demonstrate a decrease in tubulin polymerization (Chen et al., 2007).

Another important aspect in the modulation of microtubule dynamics is tubulin acetylation. This posttranslational modification occurs on Lys40 residue of α-tubulin and plays an important role in stabilizing microtubules in most cells (Topalidou et al., 2012). The first enzyme described to deacetylate α-tubulin was HDAC6 (Hubbert et al., 2002). This

enzyme has recently been linked to the process of autophagic clearance in neurodegenerative disorders. Indeed, as previously mentioned HDAC6 has a central role in misfolded and ubiquitinated protein clearance (Lee et al., 2010a). Lee et al. described the involvement of this enzyme in the fusion of the autophagosome with the lysosome and to the HDAC6-dependent step in autophagy (Lee et al., 2010a). Moreover, in the *Drosophila melanogaster* model of the neurodegenerative disease spinobulbar muscular atrophy, increasing HDAC6 expression rescues proteasome function by inducing autophagy in an HDAC6-dependent manner (Pandey et al., 2007). In cell culture, it was shown that HDAC6 binds ubiquitinated form of DJ-1 to facilitate the microtubule-dependent transport for the formation of aggresome (Olzmann et al., 2007). Furthermore, it was also seen that HDAC6 also contributes to the transport of Parkin, an E3 ubiquitin ligase, to form the aggresome (Jiang et al., 2008).

North et al. (2003) reported that a Sirtuin-2 (SIRT2) is a class III histone deacetylase that deacetylates tubulin dependent on NAD^+ as a cofactor (North et al., 2003). The inhibition of SIRT2 prevented ASYN cytotoxicity in PD models. These studies in *D. melanogaster* and rat primary dopamine-positive neurons showed that SIRT2 inhibition rescued dopaminergic degeneration (Outeiro et al., 2007). Our own data show that an increase in α-tubulin acetylation by SIRT2 inhibition improves microtubule-dependent intracellular traffic and improves autophagy (Esteves et al., unpublished data).

Lysosomal Dependency

Increasing evidence indicates that impairment of lysosomal function may contribute to the pathogenesis of several neurodegenerative diseases, including PD. It was reported that a reduction of lysosomal-associated proteins and accumulation of several autophagosomes in postmortem brain samples from patients with PD and peripheral models of PD (Dehay et al., 2010). In addition, it was observed a decrease efficacy of lysosomal-mediated clearance of autophagic vacuoles in cultured dopaminergic neurons generated from skin fibroblasts of sporadic and genetic PD patients (Sanchez-Danes et al., 2012).

Studies have reported the association between glucocerebrosidase (GBA) mutations and PD, indeed it has been described a high incidence of this mutation in sporadic PD constituting a major risk factor (Siebert et al., 2014). Further evidences demonstrated a significant decrease in the activity of GBA in the CNS especially in the SN of sporadic PD brains (Gegg et al., 2012). The enzyme GBA catalyzes the breakdown glucocerebroside into glucose and ceramide inside lysosomes and when partially defective can compromise lysosomal function, namely, in postmitotic cells, such as neurons. In addition, it has also been reported that increased mitochondrial dysfunction and oxidative stress, both involved in PD pathology, contributes to reducing the GBA protein levels and activity in SH-SY5Y cells (Gegg et al., 2012). More recently, Murphy et al. (2014) confirmed the reduction of the activity of this enzyme is not due to mutations in postmortem brains of PD patients but correlates with the accumulation of ASYN.

Another gene, that cause familial Kufor–Rakeb syndrome characterized by parkinsonism, identified as a possible responsible for lysosomal dysfunction is *ATP13A2/PARK9*. ATP13A2 is lysosomal ATPase involved in keeping lysosomal homeostasis and pH (Ramonet et al., 2012). In postmortem PD nigral samples, a reduction in ATP13A2 levels was observed and

has been proven that ATP13A2 rescues the proteolytic activity of lysosome in PD-derived fibroblasts (Dehay et al., 2012).

In various models it was found that the loss of ATP13A2 leads to lysosomal pH changes and impairment of Cathepsin D activity and consequently accumulation of autophagic vesicles (Dehay et al., 2012). Moreover, studies in primary dopaminergic cell cultures and in *Caenorhabditis elegans* have shown that this enzyme plays an important role in protection against ASYN misfolding and toxicity (Usenovic et al., 2012). Proteins associated to familial forms of PD, such as ASYN and LRRK2, are also involved in proteolytic activity of lysosome. ASYN accumulation in PD is also described not only as a consequence but also a cause of impaired proteolytic activity. Studies have shown that the expression of ASYN mutant leads to a decrease in lysosomal acidification but also was observed an inhibition of intracellular trafficking of lysosomal GBA decreasing its activity in several models of PD (Siebert et al., 2014). LRRK2 acts on NAADP, a lysosomal Ca^{2+}-mobilizing messenger nicotinic acid adenine dinucleotide phosphate receptor, that leads to lysosomal acidification (Hockey et al., 2015). It was seen that NAADP overexpression or NAADP analog can restore lysosomal pH in LRRK2 mutant cells (Gomez-Suaga et al., 2012).

AUTOPHAGY AS A THERAPEUTIC STRATEGY

A basal level of autophagy is important for intracellular homeostasis and quality control in healthy individuals. However, mounting evidence has demonstrated that disruption of its physiological function plays a role in several human diseases, including cancer, liver and heart diseases, aging, myopathies, metabolic diseases, infectious diseases, and neurodegenerative diseases, such as PD, which involve aberrant accumulation of protein aggregates and dysfunctional organelles. Interestingly, the majority of proteins associated with neurodegenerative diseases that form intracytoplasmatic aggregates are autophagic substrates (Rubinsztein et al., 2012). When autophagy is impaired the clearance of these proteins is delayed, whereas their degradation is enhanced when autophagy is upregulated. Hence targeting the autophagic pathway may be interesting in the pursue of novel and effective therapeutics for PD. Autophagy defects can occur at different stages of the pathway such as, defects on autophagosome formation, autophagosome trafficking, autophagosome/lysosome fusion, and lysosomal impairment. Therefore this must be taken into account when developing autophagy-based treatment strategies.

For quite some time owing to the protective role of autophagy its upregulation has been thought to be a therapeutic avenue. Indeed, the upregulation of autophagy may improve the clearance of neurotoxic aggregate-prone proteins such as ASYN aggregates in PD. For instance, the inhibition of mTOR, a negative regulator of autophagy with rapamycin or CCI-779, was shown to be protective in models of Huntington disease and tauopathies (Ravikumar et al., 2004). In addition, rapamycin-induced autophagy accelerates the degradation of several misfolded proteins, including ASYN (Sarkar and Rubinsztein, 2008). Another study also demonstrated that rapamycin prevented the loss of tyrosine hydroxylase following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment (Liu et al., 2013). As well, Malagelada et al. (2010) also observed the capacity of rapamycin to provide neuroprotection in several models of PD. In this study, the neuroprotective actions of rapamycin in

PD models resulted from a blockade of mTORC1-dependent translation of the pro-cell death protein RTP801 (also known as REDD1 and DDIT4). Furthermore, rapamycin also alleviated rotenone-induced mitochondrial dysfunction, apoptosis, and accumulation of ubiquitinated proteins through autophagy enhancement (Pan et al., 2009). In *D. melanogaster* exposed to paraquat, rapamycin was shown to increase the survival by inducing autophagy (Bjedov et al., 2010).

However, mTOR is required for various cellular events, including cell growth, cell proliferation, and cytoskeletal reorganization, therefore its inhibition causes some adverse effects such as cell cycle arrest. Indeed, in cell culture models of PD, blockade of mTOR leads to dopaminergic cell death (Choi et al., 2010). Moreover, loss of mTOR activity via activation of the 4EBP1 pathway by LRRK2 can also result in the loss of dopaminergic neurons (Imai et al., 2008). Interestingly, small molecule enhancers of rapamycin have been shown to induce autophagy in an mTOR-independent mechanism providing an exciting therapeutic opportunity. These molecules have been shown to enhance the elimination of mutant huntingtin and ASYN (Sarkar et al., 2007b). In fact, autophagic upregulation decreases the toxic accumulation of mutant ASYN and preserves dopaminergic neurons in several models of PD (Crews et al., 2010). In 2008, a novel group of drugs were described, namely, L-type Ca^{2+} channel antagonists (verapamil, loperamide), K^{+}ATP channel opener minoxidil, and Gi signaling activator clonidine that induce autophagy in a mTOR-independent manner (Williams et al., 2008). These compounds have been shown to enhance the clearance of mutated A53T ASYN (Williams et al., 2008).

Inositol-lowering compounds, such as lithium and valproic acid, have also effects on autophagy by reducing the levels of inositol and IP3 and have been shown to improve the clearance of mutant huntingtin and AYN (Sarkar and Rubinsztein, 2008). Kim et al. (2011) observed that lithium prevented ASYN accumulation as well as neurodegeneration in ASYN-overexpressing *in vitro* and *in vivo* models of PD (Kim et al., 2011). Moreover, valproic acid administration prevented the decrease of tyrosine hydroxylase positive neurons in the SN and striatum and ASYN alterations caused by rotenone (Monti et al., 2010). Additionally, valproate combined with lithium in MPTP-treated mice rescued dopaminergic neurons loss (Li et al., 2013).

Resveratrol is a polyphenol present in a variety of plants and has been shown to exert neuroprotective and antiaging effects (Sun et al., 2010). Interestingly, this polyphenol promoted autophagic flux and prevented rotenone-induced neuronal apoptosis *in vitro* (Lin et al., 2014). Resveratrol seems to activate autophagy via sirtuin-1 modulation, in fact resveratrol is a sirtuin-1 agonist. Notably, sirtuin-1 is a NAD^{+}-dependent histone deacetylase that has been suggested to play a role in longevity.

Additionally, it was demonstrated that the disaccharide trehalose that has the properties of a chemical chaperone induced mTOR-independent autophagy and as a result reduced ASYN and huntingtin aggregation and toxicity (Sarkar and Rubinsztein, 2008). Moreover, the use of trehalose together with rapamycin showed an additive effect on the clearance of misfolded and toxic proteins by increasing autophagic activity (Sarkar et al., 2007a). It was observed that trehalose promoted the degradation of A53T ASYN (Yu et al., 2012). The fact that trehalose reduces the aggregation of misfolding-prone proteins together with the lack of toxicity makes it a rousing therapeutic opportunity.

Additional interesting therapeutic strategy that deserves further study should include agents that increase the selective degradation of defective mitochondria rather than general

autophagy which could maximize clinical efficiency by improving selectivity. Interestingly, rapamycin enhanced mitophagy and triggered the selection of impaired mitochondria harboring a heteroplasmic mtDNA mutation in a cybrid cell model (Dai et al., 2014).

Moreover, potential drugs targeting mitochondrial dynamics are also promising, since mitochondrial fission is essential for mitophagy to occur. In fact, a small molecule that inhibits mitochondrial fission named mdivi-1 was demonstrated to inhibit mitochondrial membrane permeabilization and apoptosis in mammalian cells (Su et al., 2010). Also Bax-interacting factor 1 known to be involved in the regulation of autophagy and mitochondrial morphology was shown to be a possible therapeutic target for PD (Wang et al., 2014). Taking into account that excessive mitochondrial fragmentation occurs in sporadic PD, these could be potential therapeutics for PD treatment (Esteves et al., 2014b; Santos et al., 2014).

However, in PD it has been recently suggested that the initial steps of autophagy are not compromised whereas autophagosome clearance is defective leading to a decreased proteolytic flux of ASYN and other autophagic substrates, such as dysfunctional mitochondria (Arduino et al., 2012; Sanchez-Danes et al., 2012). As a result enhancing autophagosome formation will not improve autophagic substrate degradation and may exacerbate the accumulation of autophagic vacuoles filled with dysfunctional mitochondria and ASYN aggregates. On the other hand, in diseases such as frontotemporal dementia where autophagosome production is excessive, small molecules that inhibit autophagy such as 3-methyladenine might be promising therapeutic strategies (Lee and Gao, 2009). Hence, drugs that improve the late stages of the autophagy process may be the best therapeutic avenue to modulate autophagy in PD.

Work from our group demonstrated that in PD axonal trafficking is compromised and results from an energetic and metabolic failure leading to a deficient mobilization and fusion of autophagosomes with lysosomes and consequently to autophagic vacuoles accumulation (Arduino et al., 2012; Esteves et al., 2014a). Therefore, one possible therapeutic avenue would be to modulate autophagic vacuoles trafficking. In fact, several microtubule stabilizing drugs, such as Taxol, NAP, and Epothilone D have been shown to improve microtubule network assembly and boost autophagic flux in PD models (Cartelli et al., 2013; Esteves et al., 2009, 2014a). Indeed, autophagic flux is highly dependent on microtubule network.

More interestingly, acetylated microtubules are required for the fusion between autophagosomes and lysosomes and tubulin acetylation, a marker of stable microtubules is in fact decreased in PD (Esteves et al., 2014a; Outeiro et al., 2007). Hence, histone deacetylases inhibitors have also been suggested to be promising targets for therapeutic interventions in neurodegenerative disorders in which accumulation of protein aggregates are hallmarks, such as PD. In fact, a study from 2009 revealed that acetylation facilitates trafficking of mutant huntingtin into autophagosomes improving clearance of the mutant protein by autophagy, suggesting a critical role for acetylation in the clearance regulation of this protein (Jeong et al., 2009).

Lysosomal dysfunction has been implicated in PD however it does not seem to be a direct cause of PD (Cheung and Ip, 2011). Interestingly, heterozygous mutations in the *GBA* gene which encode the lysosomal GBA are a genetic risk factor for PD, and reduced GBA accumulation was observed in PD models (Murphy and Halliday, 2014). Furthermore, mutations in the lysosomal membrane protein ATP13A2 cause familial Kufor–Rakeb syndrome

characterized by parkinsonism (Usenovic et al., 2012). Several secondary pathologic hallmarks such as alterations of intracellular trafficking and impairment of autophagy that are associated with lysosomal storage disorders are recapitulated in PD (Bellettato and Scarpa, 2010). As well, a number of lysosomal markers were significantly reduced in postmortem brain samples from patients with PD indicating lysosomal deficiency (Chu et al., 2009). Therefore, therapeutic targets that aim to improve lysosomal function may also be exciting opportunities for the treatment of PD. Indeed, in Alzheimer's disease, lysosomal proteolysis modulation has proven to promote the clearance of peptides Aβ and to have promising effects on neuronal and cognitive function (Steele et al., 2013). Remarkably, it was suggested that rapamycin had neuroprotective effects via induction of lysosome-mediated autophagic degradation. Indeed, rapamycin restored the dysfunctional lysosome-mediated autophagosome clearance and attenuated dopaminergic nigrostriatal degeneration in a MPTP model of PD (Dehay et al., 2010). This indicates that rapamycin other than increasing the autophagosome formation has also a role in lysosomal biogenesis augmenting the number of functional lysosomes that will fuse with filled autophagosomes (Bove et al., 2011). Combining a therapy that improves autophagosomes mobilization toward lysosomes and lysosomal efficiency would be interesting and likely to deliver exciting results for the treatment of PD.

Overall, understanding specifically where autophagy is defective in neurodegenerative diseases will help to design more promising and effective therapies.

CONCLUDING REMARKS

Autophagy is an evolutionarily-conserved and genetically-regulated process and one of the major routes for the bulk degradation of long-lived cytosolic proteins and organelles. It is an important intracellular catabolic mechanism either in normal or pathophysiological conditions. Importantly, it is involved in several physiological processes including cell survival, cell quality control and homeostasis, differentiation, development as well as regulation of inflammatory responses, antigen presentation, and pathogen clearance. In response to certain stimuli such as starvation, endoplasmic reticulum stress, mitochondrial stress, hypoxia, accumulation of protein aggregates, and microbial infection, autophagy is activated to promote cell survival. Remarkably, autophagy is known to be impaired in many human diseases in which the accumulation of aberrant protein aggregates occurs.

In PD, autophagic dysfunction has long been implicated in the death of dopaminergic neurons in the SNpc. Indeed, the misfolding and aggregation of ASYN in LBs that are not degraded by macroautophagy and accumulate in the cytosol are known to disrupt the normal neuronal function and to culminate in neuronal death.

In the last few years, macroautophagy has gained much more attention and is now seen as a selective rather that a random process. For instance, mitophagy selectively engulfs dysfunctional mitochondria by autophagosomes for subsequent degradation. Taking into account that mitochondria dynamics and function is impaired in PD (Esteves et al., 2009; Santos et al., 2014), future treatment strategies that selectively target the degradation of dysfunctional mitochondria by mitophagy are likely to provide interesting outcomes. Furthermore elucidating the pathway PINK1–Parkin–PARL will be also of great interest to better understand the underlying mechanisms of mitophagy and how it is affected in PD.

Nevertheless accumulating evidence suggests that the accumulation of autophagosomes filled with dysfunctional mitochondria and ASYN inclusions occur due to impaired autophagic vesicles trafficking and clearance (Arduino et al., 2012). This indicates a problem at the level of autophagosome trafficking, autophagosome/lysosome fusion, and/or lysosomal deficiency. Indeed, as previously mentioned microtubule dynamics/trafficking and lysosomal-associated proteins function has been shown to be defective in PD (Dehay et al., 2010; Esteves et al., 2014a).

Over the years several therapeutic strategies exploring autophagy have been selected for clinical trials. However, so far none has proved to yield promising results in the treatment of PD. This is probably due to the fact that the majority of these therapeutic strategies are not specific and increase bulk autophagy rather than selective autophagy. Moreover, as previously mentioned if the problem lies in autophagosome trafficking and clearance, autophagic stimulation will further increase the formation and accumulation of build-up autophagosomes.

In light with this it is important to further dissect specifically how autophagy is affected in both familial and sporadic PD to more efficiently develop novel therapeutic strategies. Moreover one interesting hypothesis would be to combine multiple strategies that target not only lysosomal function but also axonal trafficking in order to accomplish better results that hopefully will in the end slow down or halt the development of PD.

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References

Altman, B.J., Rathmell, J.C., 2012. Metabolic stress in autophagy and cell death pathways. Cold Spring Harb. Perspect. Biol. 4, a008763.

Alvarez-Erviti, L., Rodriguez-Oroz, M.C., Cooper, J.M., et al., 2010. Chaperone-mediated autophagy markers in Parkinson disease brains. Arch. Neurol. 67, 1464–1472.

Arduino, D.M., Esteves, A.R., Cortes, L., et al., 2012. Mitochondrial metabolism in Parkinson's disease impairs quality control autophagy by hampering microtubule-dependent traffic. Hum. Mol. Genet. 21, 4680–4702.

Arduino, D.M., Esteves, A.R., Cardoso, S.M., 2013. Mitochondria drive autophagy pathology via microtubule disassembly: a new hypothesis for Parkinson disease. Autophagy 9, 112–114.

Banerjee, R., Beal, M.F., Thomas, B., 2010. Autophagy in neurodegenerative disorders: pathogenic roles and therapeutic implications. Trends Neurosci. 33, 541–549.

Bellettato, C.M., Scarpa, M., 2010. Pathophysiology of neuropathic lysosomal storage disorders. J. Inherit. Metab. Dis. 33, 347–362.

Bjedov, I., Toivonen, J.M., Kerr, F., et al., 2010. Mechanisms of life span extension by rapamycin in the fruit fly *Drosophila melanogaster*. Cell Metab. 11, 35–46.

Bove, J., Martinez-Vicente, M., Vila, M., 2011. Fighting neurodegeneration with rapamycin: mechanistic insights. Nat. Rev. Neurosci. 12, 437–452.

Carballo-Carbajal, I., Weber-Endress, S., Rovelli, G., et al., 2010. Leucine-rich repeat kinase 2 induces alpha-synuclein expression via the extracellular signal-regulated kinase pathway. Cell Signal. 22, 821–827.

Cardoso, S.M., 2011. The mitochondrial cascade hypothesis for Parkinson's disease. Curr. Pharm. Des. 17, 3390–3397.

Cartelli, D., Casagrande, F., Busceti, C.L., et al., 2013. Microtubule alterations occur early in experimental parkinsonism and the microtubule stabilizer epothilone D is neuroprotective. Sci. Rep. 3, 1837.

Chen, L., Jin, J., Davis, J., et al., 2007. Oligomeric alpha-synuclein inhibits tubulin polymerization. Biochem. Biophys. Res. Commun. 356, 548–553.
Cheung, Z.H., Ip, N.Y., 2011. Autophagy deregulation in neurodegenerative diseases—recent advances and future perspectives. J. Neurochem. 118, 317–325.
Choi, K.C., Kim, S.H., Ha, J.Y., et al., 2010. A novel mTOR activating protein protects dopamine neurons against oxidative stress by repressing autophagy related cell death. J. Neurochem. 112, 366–376.
Chu, Y., Dodiya, H., Aebischer, P., et al., 2009. Alterations in lysosomal and proteasomal markers in Parkinson's disease: relationship to alpha-synuclein inclusions. Neurobiol. Dis. 35, 385–398.
Chu, Y., Morfini, G.A., Langhamer, L.B., et al., 2012. Alterations in axonal transport motor proteins in sporadic and experimental Parkinson's disease. Brain 135, 2058–2073.
Cook, C., Stetler, C., Petrucelli, L., 2012. Disruption of protein quality control in Parkinson's disease. Cold Spring Harb. Perspect. Med. 2, a009423.
Cordero, M.D., De Miguel, M., Moreno Fernandez, A.M., et al., 2010. Mitochondrial dysfunction and mitophagy activation in blood mononuclear cells of fibromyalgia patients: implications in the pathogenesis of the disease. Arthritis Res. Ther. 12, R17.
Crews, L., Spencer, B., Desplats, P., et al., 2010. Selective molecular alterations in the autophagy pathway in patients with Lewy body disease and in models of alpha-synucleinopathy. PLoS One 5, e9313.
Cuervo, A.M., Stefanis, L., Fredenburg, R., et al., 2004. Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy. Science 305, 1292–1295.
Dagda, R.K., Cherra III, S.J., Kulich, S.M., et al., 2009. Loss of PINK1 function promotes mitophagy through effects on oxidative stress and mitochondrial fission. J. Biol. Chem. 284, 13843–13855.
Dai, Y., Zheng, K., Clark, J., et al., 2014. Rapamycin drives selection against a pathogenic heteroplasmic mitochondrial DNA mutation. Hum. Mol. Genet. 23, 637–647.
Damme, M., Suntio, T., Saftig, P., et al., 2014. Autophagy in neuronal cells: general principles and physiological and pathological functions. Acta Neuropathol. 129(3), 337–362.
Dehay, B., Bove, J., Rodriguez-Muela, N., et al., 2010. Pathogenic lysosomal depletion in Parkinson's disease. J. Neurosci. 30, 12535–12544.
Dehay, B., Ramirez, A., Martinez-Vicente, M., et al., 2012. Loss of P-type ATPase ATP13A2/PARK9 function induces general lysosomal deficiency and leads to Parkinson disease neurodegeneration. Proc. Natl. Acad. Sci. U.S.A. 109, 9611–9616.
Distelmaier, F., Valsecchi, F., Liemburg-Apers, D.C., et al., 2015. Mitochondrial dysfunction in primary human fibroblasts triggers an adaptive cell survival program that requires AMPK-alpha. Biochim. Biophys. Acta 1852, 529–540.
Ebrahimi-Fakhari, D., Wahlster, L., McLean, P.J., 2012. Protein degradation pathways in Parkinson's disease: curse or blessing. Acta Neuropathol. 124, 153–172.
Esteves, A.R., Arduino, D.M., Swerdlow, R.H., et al., 2009. Oxidative stress involvement in alpha-synuclein oligomerization in Parkinson's disease cybrids. Antioxid. Redox Signal. 11, 439–448.
Esteves, A.R., Arduino, D.M., Swerdlow, R.H., et al., 2010. Microtubule depolymerization potentiates alpha-synuclein oligomerization. Front. Aging Neurosci. 1, 5.
Esteves, A.R., Gozes, I., Cardoso, S.M., 2014a. The rescue of microtubule-dependent traffic recovers mitochondrial function in Parkinson's disease. Biochim. Biophys. Acta 1842, 7–21.
Esteves, A.R., G-Fernandes, M., Santos, D., et al., 2014b. The Upshot of LRRK2 inhibition to Parkinson's disease paradigm. Mol. Neurobiol.
Fecto, F., Esengul, Y.T., Siddique, T., 2014. Protein recycling pathways in neurodegenerative diseases. Alzheimers Res. Ther. 6, 13.
Furuta, N., Fujita, N., Noda, T., et al., 2010. Combinational soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor proteins VAMP8 and Vti1b mediate fusion of antimicrobial and canonical autophagosomes with lysosomes. Mol. Biol. Cell 21, 1001–1010.
Gegg, M.E., Burke, D., Heales, S.J., et al., 2012. Glucocerebrosidase deficiency in substantia nigra of Parkinson disease brains. Ann. Neurol. 72, 455–463.
Ghavami, S., Shojaei, S., Yeganeh, B., et al., 2014. Autophagy and apoptosis dysfunction in neurodegenerative disorders. Prog. Neurobiol. 112, 24–49.
Gomez-Suaga, P., Luzon-Toro, B., Churamani, D., et al., 2012. Leucine-rich repeat kinase 2 regulates autophagy through a calcium-dependent pathway involving NAADP. Hum. Mol. Genet. 21, 511–525.

Hara, T., Nakamura, K., Matsui, M., et al., 2006. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature 441, 885–889.
Hockey, L.N., Kilpatrick, B.S., Eden, E.R., et al., 2015. Dysregulation of lysosomal morphology by pathogenic LRRK2 is corrected by TPC2 inhibition. J. Cell Sci. 128, 232–238.
Hubbert, C., Guardiola, A., Shao, R., et al., 2002. HDAC6 is a microtubule-associated deacetylase. Nature 417, 455–458.
Imai, Y., Gehrke, S., Wang, H.Q., et al., 2008. Phosphorylation of 4E-BP by LRRK2 affects the maintenance of dopaminergic neurons in *Drosophila*. EMBO J. 27, 2432–2443.
Jahreiss, L., Menzies, F.M., Rubinsztein, D.C., 2008. The itinerary of autophagosomes: from peripheral formation to kiss-and-run fusion with lysosomes. Traffic 9, 574–587.
Jeong, H., Then, F., Melia Jr., T.J., et al., 2009. Acetylation targets mutant huntingtin to autophagosomes for degradation. Cell 137, 60–72.
Jiang, Q., Ren, Y., Feng, J., 2008. Direct binding with histone deacetylase 6 mediates the reversible recruitment of parkin to the centrosome. J. Neurosci. 28, 12993–13002.
Jin, S.M., Lazarou, M., Wang, C., et al., 2010. Mitochondrial membrane potential regulates PINK1 import and proteolytic destabilization by PARL. J. Cell Biol. 191, 933–942.
Kim, Y.H., Rane, A., Lussier, S., et al., 2011. Lithium protects against oxidative stress-mediated cell death in alpha-synuclein-overexpressing *in vitro* and *in vivo* models of Parkinson's disease. J. Neurosci. Res. 89, 1666–1675.
Kondapalli, C., Kazlauskaite, A., Zhang, N., et al., 2012. PINK1 is activated by mitochondrial membrane potential depolarization and stimulates Parkin E3 ligase activity by phosphorylating Serine 65. Open Biol. 2, 120080.
Kubli, D.A., Gustafsson, A.B., 2012. Mitochondria and mitophagy: the yin and yang of cell death control. Circ. Res. 111, 1208–1221.
Lee, H.J., Khoshaghideh, F., Patel, S., et al., 2004. Clearance of alpha-synuclein oligomeric intermediates via the lysosomal degradation pathway. J. Neurosci. 24, 1888–1896.
Lee, J.A., Gao, F.B., 2009. Inhibition of autophagy induction delays neuronal cell loss caused by dysfunctional ESCRT-III in frontotemporal dementia. J. Neurosci. 29, 8506–8511.
Lee, J.Y., Koga, H., Kawaguchi, Y., et al., 2010a. HDAC6 controls autophagosome maturation essential for ubiquitin-selective quality-control autophagy. EMBO J. 29, 969–980.
Lee, J.Y., Nagano, Y., Taylor, J.P., et al., 2010b. Disease-causing mutations in parkin impair mitochondrial ubiquitination, aggregation, and HDAC6-dependent mitophagy. J. Cell Biol. 189, 671–679.
Li, X.Z., Chen, X.P., Zhao, K., et al., 2013. Therapeutic effects of valproate combined with lithium carbonate on MPTP-induced parkinsonism in mice: possible mediation through enhanced autophagy. Int. J. Neurosci. 123, 73–79.
Lin, T.K., Chen, S.D., Chuang, Y.C., et al., 2014. Resveratrol partially prevents rotenone-induced neurotoxicity in dopaminergic SH-SY5Y cells through induction of heme oxygenase-1 dependent autophagy. Int. J. Mol. Sci. 15, 1625–1646.
Liu, K., Shi, N., Sun, Y., et al., 2013. Therapeutic effects of rapamycin on MPTP-induced Parkinsonism in mice. Neurochem. Res. 38, 201–207.
Maday, S., Holzbaur, E.L., 2014. Autophagosome biogenesis in primary neurons follows an ordered and spatially regulated pathway. Dev. Cell 30, 71–85.
Malagelada, C., Jin, Z.H., Jackson-Lewis, V., et al., 2010. Rapamycin protects against neuron death in *in vitro* and *in vivo* models of Parkinson's disease. J. Neurosci. 30, 1166–1175.
Mao, K., Klionsky, D.J., 2011. AMPK activates autophagy by phosphorylating ULK1. Circ. Res. 108, 787–788.
Martinez-Vicente, M., Talloczy, Z., Kaushik, S., et al., 2008. Dopamine-modified alpha-synuclein blocks chaperone-mediated autophagy. J. Clin. Invest. 118, 777–788.
Massey, A.C., Kaushik, S., Sovak, G., et al., 2006. Consequences of the selective blockage of chaperone-mediated autophagy. Proc. Natl. Acad. Sci. U. S. A. 103, 5805–5810.
Menzies, F.M., Moreau, K., Rubinsztein, D.C., 2011. Protein misfolding disorders and macroautophagy. Curr. Opin. Cell Biol. 23, 190–197.
Monti, B., Gatta, V., Piretti, F., et al., 2010. Valproic acid is neuroprotective in the rotenone rat model of Parkinson's disease: involvement of alpha-synuclein. Neurotox. Res. 17, 130–141.
Mortensen, M., Ferguson, D.J., Edelmann, M., et al., 2010. Loss of autophagy in erythroid cells leads to defective removal of mitochondria and severe anemia *in vivo*. Proc. Natl. Acad. Sci. U. S. A. 107, 832–837.
Murphy, K.E., Halliday, G.M., 2014. Glucocerebrosidase deficits in sporadic Parkinson disease. Autophagy 10, 1350–1351.

Murphy, K.E., Gysbers, A.M., Abbott, S.K., et al., 2014. Reduced glucocerebrosidase is associated with increased alpha-synuclein in sporadic Parkinson's disease. Brain 137, 834–848.
Narendra, D., Tanaka, A., Suen, D.F., et al., 2009. Parkin-induced mitophagy in the pathogenesis of Parkinson disease. Autophagy 5, 706–708.
Narendra, D., Walker, J.E., Youle, R., 2012. Mitochondrial quality control mediated by PINK1 and Parkin: links to parkinsonism. Cold Spring Harb. Perspect. Biol., 1, 4(11).
Narendra, D.P., Jin, S.M., Tanaka, A., et al., 2010. PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. PLoS Biol. 8, e1000298.
Nixon, R.A., 2013. The role of autophagy in neurodegenerative disease. Nat. Med. 19, 983–997.
North, B.J., Marshall, B.L., Borra, M.T., et al., 2003. The human Sir2 ortholog, SIRT2, is an NAD+ -dependent tubulin deacetylase. Mol. Cell 11, 437–444.
Okatsu, K., Kimura, M., Oka, T., et al., 2015. Unconventional PINK1 localization to the outer membrane of depolarized mitochondria drives Parkin recruitment. J. Cell Sci. 128, 964–978.
Olzmann, J.A., Li, L., Chudaev, M.V., et al., 2007. Parkin-mediated K63-linked polyubiquitination targets misfolded DJ-1 to aggresomes via binding to HDAC6. J. Cell Biol. 178, 1025–1038.
Outeiro, T.F., Kontopoulos, E., Altmann, S.M., et al., 2007. Sirtuin 2 inhibitors rescue alpha-synuclein-mediated toxicity in models of Parkinson's disease. Science 317, 516–519.
Outeiro, T.F., Putcha, P., Tetzlaff, J.E., et al., 2008. Formation of toxic oligomeric alpha-synuclein species in living cells. PLoS One 3, e1867.
Pan, T., Rawal, P., Wu, Y., et al., 2009. Rapamycin protects against rotenone-induced apoptosis through autophagy induction. Neuroscience 164, 541–551.
Pandey, U.B., Nie, Z., Batlevi, Y., et al., 2007. HDAC6 rescues neurodegeneration and provides an essential link between autophagy and the UPS. Nature 447, 859–863.
Ramonet, D., Podhajska, A., Stafa, K., et al., 2012. PARK9-associated ATP13A2 localizes to intracellular acidic vesicles and regulates cation homeostasis and neuronal integrity. Hum. Mol. Genet. 21, 1725–1743.
Ravikumar, B., Vacher, C., Berger, Z., et al., 2004. Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. Nat. Genet. 36, 585–595.
Rogov, V., Dotsch, V., Johansen, T., et al., 2014. Interactions between autophagy receptors and ubiquitin-like proteins form the molecular basis for selective autophagy. Mol. Cell 53, 167–178.
Rubinsztein, D.C., Codogno, P., Levine, B., 2012. Autophagy modulation as a potential therapeutic target for diverse diseases. Nat. Rev. Drug Discov. 11, 709–730.
Sanchez-Danes, A., Richaud-Patin, Y., Carballo-Carbajal, I., et al., 2012. Disease-specific phenotypes in dopamine neurons from human iPS-based models of genetic and sporadic Parkinson's disease. EMBO Mol. Med. 4, 380–395.
Santos, D., Esteves, A.R., Silva, D.F., et al., 2014. The impact of mitochondrial fusion and fission modulation in sporadic Parkinson's disease. Mol. Neurobiol. 52(1), 573–586.
Sarkar, S., Rubinsztein, D.C., 2008. Small molecule enhancers of autophagy for neurodegenerative diseases. Mol. Biosyst. 4, 895–901.
Sarkar, S., Davies, J.E., Huang, Z., et al., 2007a. Trehalose, a novel mTOR-independent autophagy enhancer, accelerates the clearance of mutant huntingtin and alpha-synuclein. J. Biol. Chem. 282, 5641–5652.
Sarkar, S., Perlstein, E.O., Imarisio, S., et al., 2007b. Small molecules enhance autophagy and reduce toxicity in Huntington's disease models. Nat. Chem. Biol. 3, 331–338.
Sarkar, S., Ravikumar, B., Floto, R.A., et al., 2009. Rapamycin and mTOR-independent autophagy inducers ameliorate toxicity of polyglutamine-expanded huntingtin and related proteinopathies. Cell Death Differ. 16, 46–56.
Schon, E.A., Przedborski, S., 2011. Mitochondria: the next (neurode)generation. Neuron 70, 1033–1053.
Siebert, M., Sidransky, E., Westbroek, W., 2014. Glucocerebrosidase is shaking up the synucleinopathies. Brain 137, 1304–1322.
Spencer, B., Potkar, R., Trejo, M., et al., 2009. Beclin 1 gene transfer activates autophagy and ameliorates the neurodegenerative pathology in alpha-synuclein models of Parkinson's and Lewy body diseases. J. Neurosci. 29, 13578–13588.
Steele, J.W., Lachenmayer, M.L., Ju, S., et al., 2013. Latrepirdine improves cognition and arrests progression of neuropathology in an Alzheimer's mouse model. Mol. Psychiatry 18, 889–897.
Su, B., Wang, X., Bonda, D., et al., 2010. Abnormal mitochondrial dynamics—a novel therapeutic target for Alzheimer's disease? Mol. Neurobiol. 41, 87–96.

Sun, A.Y., Wang, Q., Simonyi, A., et al., 2010. Resveratrol as a therapeutic agent for neurodegenerative diseases. Mol. Neurobiol. 41, 375–383.
Takano-Ohmuro, H., Mukaida, M., Kominami, E., et al., 2000. Autophagy in embryonic erythroid cells: its role in maturation. Eur. J. Cell. Biol. 79, 759–764.
Thomas, K.J., McCoy, M.K., Blackinton, J., et al., 2011. DJ-1 acts in parallel to the PINK1/parkin pathway to control mitochondrial function and autophagy. Hum. Mol. Genet. 20, 40–50.
Topalidou, I., Keller, C., Kalebic, N., et al., 2012. Genetically separable functions of the MEC-17 tubulin acetyltransferase affect microtubule organization. Curr. Biol. 22, 1057–1065.
Usenovic, M., Tresse, E., Mazzulli, J.R., et al., 2012. Deficiency of ATP13A2 leads to lysosomal dysfunction, alpha-synuclein accumulation, and neurotoxicity. J. Neurosci. 32, 4240–4246.
Wang, D.B., Uo, T., Kinoshita, C., et al., 2014. Bax interacting factor-1 promotes survival and mitochondrial elongation in neurons. J. Neurosci. 34, 2674–2683.
Williams, A., Sarkar, S., Cuddon, P., et al., 2008. Novel targets for Huntington's disease in an mTOR-independent autophagy pathway. Nat. Chem. Biol. 4, 295–305.
Wong, A.S., Cheung, Z.H., Ip, N.Y., 2011. Molecular machinery of macroautophagy and its deregulation in diseases. Biochim. Biophys. Acta 1812, 1490–1497.
Wu, S.B., Wu, Y.T., Wu, T.P., et al., 2014. Role of AMPK-mediated adaptive responses in human cells with mitochondrial dysfunction to oxidative stress. Biochim. Biophys. Acta 1840, 1331–1344.
Yang, Q., She, H., Gearing, M., et al., 2009. Regulation of neuronal survival factor MEF2D by chaperone-mediated autophagy. Science 323, 124–127.
Yu, W.B., Jiang, T., Lan, D.M., et al., 2012. Trehalose inhibits fibrillation of A53T mutant alpha-synuclein and disaggregates existing fibrils. Arch. Biochem. Biophys. 523, 144–150.
Zhou, R.M., Huang, Y.X., Li, X.L., et al., 2010. Molecular interaction of alpha-synuclein with tubulin influences on the polymerization of microtubule *in vitro* and structure of microtubule in cells. Mol. Biol. Rep. 37, 3183–3192.

CHAPTER

12

Role of Autophagy in Cancer Development via Mitochondrial Reactive Oxygen Species

Bo Liu and Gu He

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Abstract

Mysterious transition and molecular mechanisms of autophagy have recently caused disturbance and obstacle in cancer diagnosis and therapy. By means of the nonselective and selective autophagy, cancer cells maintain their proliferative rates or endure cell death executors. So far, free radicals have been identified in the complex metabolism, which has a complex relationship with autophagy. The dual role of autophagy regulates either deleterious or beneficial role of reactive oxygen species (ROS) to maintain cell metabolism and shows quite unusual state in cancer. Mitochondrial ROS (mitoROS) is the main source of endogenous oxygen free radicals, and it may mediate autophagy by multiple pathways such as PI3K–Akt–mTOR pathway, MAPKs, and some transcription regulators. On the contrary, it may also be regulated by selective mitophagy. The interconversion between cytoprotective and cytotoxic roles of autophagy conundrum may be solved by untangling the knot between mitoROS and autophagy. Thus, manipulation of ROS generation and removal may lead to future cancer drug discovery, and the understanding of how drug resistance is involved in cancer therapy.

INTRODUCTION

Free radicals are highly unstable and reactive chemical species with one or two unpaired electrons. The major free radicals in biologic systems are reactive oxygen species (ROS), which participate in countless reactions and can be generated in distinct organelles. During normal homeostasis, endogenous ROS are mainly formed through Ero1–DPI oxidative folding system in endoplasmic reticulum (ER), nicotinamide adenine dinucleotide phosphate oxidases (NOX) complex at the membrane, and the mitochondrial electron transport chain (ETC), in which mitochondrial ROS (mitoROS) is the major source of ROS.

Autophagy is a lysosome-dependent cellular degradation process, and deletions of the essential autophagy genes exhibit various pathological conditions, though direct cause for these phenotypes is not clear. Autophagy is widely distributed and widespread in tissues at low level under nonstress conditions and can be triggered by numerous stresses such as nutrient starvation, hypoxia, energy stress, intracellular ROS levels, chemical drugs, and other stimuli. There are normally three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy, during which macroautophagy (hereafter called autophagy) is most characterized and commonly observed one. Until now, more than 40 proteins have been identified to be involved in such processes. The induction of autophagy is mediated by several signaling cascades, and ULK1/2-mAtg13-FIP200-Atg101 is a critical one. The class III phosphatidylinositol 3-kinase (PI3K CIII) complex, consisting of Vps34 (the PtdIns3K), Vps15, Atg14, and Beclin-1, functions in the formation of phagophore. In phagophore elongation, phagophore elongation requires two ubiquitin-like conjugation complexes, one is Atg12–Atg5–Atg16 complex, which associates with phagophore. The other is LC3–phosphatidylethanolamin (PE) conjugation (after processed by Atg4, Atg3, and Atg7) that exposes a glycine residue and conjugates with PE. Moreover, after autophagosome is formed, Atg4 recycles LC3 via deconjugation of LC3–PE. Then this process requires cargo adapter proteins; for instance, Sequestosome 1(SQSTM1)/p62, neighbor of *BRCA1* gene 1 (NBR1) or Bcl-2/adenovirus E1B 19-kDa-interacting protein 3 long form (NIX/BNIP3L), to interact with LC3 and ubiquitinated cargo for the recruitment into the autophagosomes. Nonselective autophagy may target long-lived proteins, selective autophagy may degrade damaged organelles, such as peroxisome, mitochondria, and ER, called pexophagy, mitophagy, and reticulophagy, respectively. Elimination of damaged

mitochondria by autophagy leads to decreased ROS production, thereby limiting tumor-promoting effect of ROS. To note, scAtg32 is a mitochondrial outer-membrane proteins that binds to Atg11 as a receptor during mitophagy. BNIP3 is also a receptor on mitochondria and mediates mitophagy through interaction with the LC3 N-terminal regions (Bhogal et al., 2012).

MITOCHONDRIA AND mitoROS

Mitochondrion Function

Mitochondria are intracellular organelles responsible for cellular metabolism, including Ca^{2+} homeostasis, cell death signaling, and especially function of energy transduction process leading to ATP synthesis. Mitochondria are characterized by two structurally and functionally distinct membrane architectures: the mitochondrial outer membrane and the inner membrane. These two membranes separate the cytosol from the mitochondrial matrix. Also, the intermembrane space (IMS) is a small aqueous space located between the two membranes. Mitochondria are considered of being pivotal in triggering cell death via disruption of mitochondrial ETC and energy metabolism, as well as release of apoptosis-related IMS proteins, such as cytochrome c (cyto c) and AIF. Those IMS proteins may undergo cytotoxic activities after discharging from the IMS into the cytoplasm, thus culminating in cell death (Müller et al., 2015).

mitoROS Generation

mitoROS is the major source of oxygen radicals, including superoxide ($O_2^{-\bullet}$), hydroxyl radical ($OH^{\bullet}$), nitric oxide (NO), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$). ROS are short-lived with a tendency to form other species of ROS by reacting with various biomolecules. Both exogenous and endogenous ROS are involved in the regulation of many biologic processes and are mediated via numerous stresses or signaling, such as signaling of growth factors and amino acids, oxidative or ER stresses, and immunity signaling. $O_2^{-\bullet}$, $OH^{\bullet}$, and H_2O_2 are the most abundant ROS in living organisms. ROS are divided into free radical ROS and highly reactive nonradical ROS, between which highly reactive nonradical ROS give rise to radical forms of ROS (Murphy, 2009).

The majority of oxygen is reduced to water within a mitochondrial cytochrome oxidase catalyzed reaction, and the mitochondrial ETC is a major source of $O_2^{-\bullet}$ formation through continuous aerobic respiration generation of $O_2^{-\bullet}$. Mitochondrial ETC contains several redox centers that may directly transfer an electron to oxygen, including five protein complexes (complexes I to V), coenzyme Q (CoQ), and cyto c. From NADH at complex I (NADH–coenzyme Q oxidoreductase), electrons are transferred to complex II (succinate-Coenzyme Q oxidoreductase) and through CoQ to complex III (CoQ-cyto c oxidoreductase) and a lipid-soluble carrier, ubiquinone (CoQ), to form ubiquinol (cyto c). Subsequently, through complex IV (cyto c oxidase), cyto c is accepted by the final acceptor, the oxygen, to produce water. In addition, reduction of electron potential energy leads to mitochondrial matrix protons release into the IMS, setting up a proton-motive force. It drives protons back

into the matrix trough complex V (ATP synthase) and results in ATP synthesis. The mitochondrial ETC protein complexs (complex I to V) compose the process of ATP synthesis, and this process is named oxidative phosphorylation (Li et al., 2011).

Under the circumstances that reverse electron transport occurs or NADH/NAD+ ratio is high, the mitochondrial complex I produces large amounts of $O_2^{-\bullet}$. Compared with complex I, complex III also produces $O_2^{-\bullet}$ but at a much lower level under physiologic conditions, and other enzymes of mitochondrial can also generate $O_2^{-\bullet}$, such as α-ketoglutarate dehydrogenase, α-glycerophosphate dehydrogenase, and dihydroorotate dehydrogenase. Besides ETC, NOXs or XOR are also sources of $O_2^{-\bullet}$ generation. During the conversion between $O_2^{-\bullet}$ and H_2O_2 by superoxide dismutase (SOD), mitochondria have their own SOD, MnSOD, to specifically function within the mitochondrial matrix, while XOR or NOXs is dismutated by cytosolic CuZnSOD (Venditti et al., 2013).

High concentration of O_2 or nonefficient elimination of $O_2^{-\bullet}$ causes oxidative stress, while ROS are also crucial secondary messengers of signal-transduction pathways, suggesting ROS are double-edged swords and critical to cell proliferation and death. The deleterious effects of ROS largely contribute to their capacity for damaging cellular components, such as DNA mutation, protein oxidation, lipid oxidation, iron channel disruption, and cell death. Excessive ROS causes cytotoxic effects and link to aging and many diseases, including cancer, cardiovascular diseases, neurodegenerative diseases, and diabetes. Nevertheless, relying on the evidence that cytokines and growth factors stimulate ROS production when they initiate signaling cascades or bind to membrane receptors, ROS are essential signaling molecules in cell proliferation. Moreover, ROS regulate kinase activities (Src, MAPKs, and Akt) and transcription factors (NF-κB) to promote cell growth. ROS can activate pathways of programmed cell death, such as apoptosis, autophagic cell death, and necroptosis, indicating their potentials in cancer therapy (Li et al., 2011).

Removal of mitoROS

To govern intracellular ROS at a tolerable level, ROS scavengers undergo redox systems to maintain redox homeostasis, including SOD, catalase (decomposes H_2O_2 into H_2O and O_2), glutathione peroxidase, and antioxidants such as glutathione (GSH). GSH converted to its oxidized form, glutathione disulfide (GSSG), coupled with many ROS-decomposition reactions and disulfide bond reduction. In addition, as the patterns for ROS and autophagy alteration are distinct in various circumstances, autophagy can regulate both generation and removal of ROS. For instance, through arsenic exposure generated steady increased ROS levels, ROS generation and autophagy are activated; subsequently, treatment via autophagy stimulation increases mitochondria turnover and decreases ROS generation. And to ameliorate the oxidative toxicity, autophagy reduces ROS level via elimination of ROS production compartments, such as mitochondria and ER. For instance, antiviral signals of cytosolic RIG-I–like receptors (RLRs) could initiate autophagy, and its deficiency leads to the accumulation of ROS and dysfunctional mitochondria (Zhang et al., 2012a,b). Furthermore, large amounts of ROS, either produced from mitochondria or other cellular sources, inactivate components of ETC and mitochondrial proteins, causing mitochondrial dysfunction. A disturbance of mitochondrial function can damage both respiration and oxidative energy production, and multiple

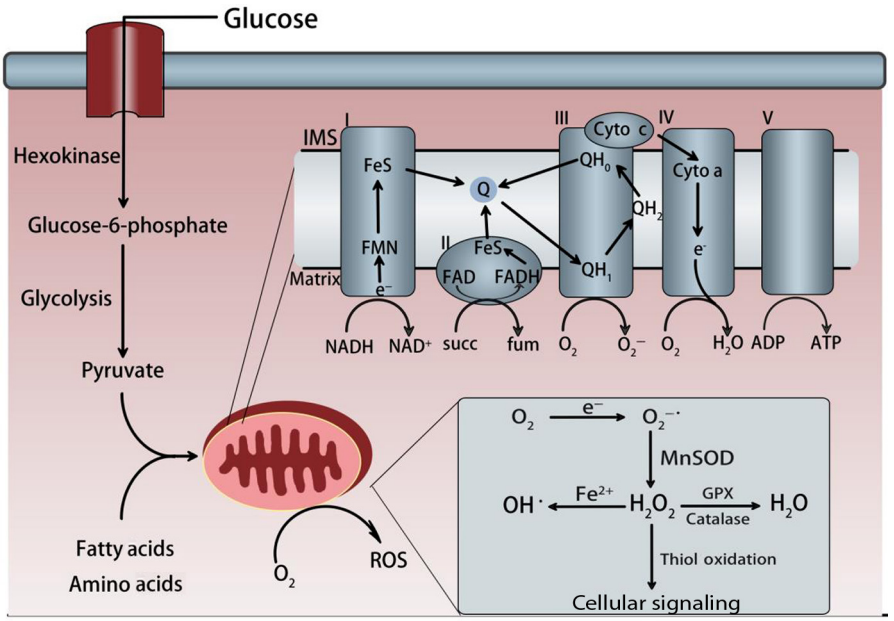


FIGURE 12.1 Generation and interconversion of mitoROS. Complex I has more than 40 subunits with one noncovalently bound FMN and eight iron–sulfur clusters, and due to its complexity, mechanisms of superoxide generation in Complex I are not elucidated. Complex I is the major source of superoxide generation, additionally, Complex III can also produce superoxide.

biosynthetic reaction pathways. Thus, evolving mechanisms to scavenge the dysfunction mitochondria is critical to maintain cellular redox homeostasis and alleviate the impact of cytotoxic ROS. Mitophagy is essential to this process by promoting turnover of dysfunctional mitochondria, although how cell distinguishes nonfunctional mitochondria remains to be elucidated. Mitophagy can be triggered by the loss of mitochondrial membrane potential (MMP) and mitochondrial fragmentation, indicating a complex regulatory mechanism between ROS and autophagy (Chourasia et al., 2015) (Figure 12.1).

RELATIONSHIP BETWEEN mitoROS AND AUTOPHAGY

Mitochondria generate most of intracellular ROS through oxidative phosphorylation, thus, elucidating that mitoROS mediation of autophagy is critical in metabolism bioreactions, aerobic glycolysis, and pathological mechanisms. ROS levels regulate autophagy both in cell survival and cell death mechanisms. ROS-mediated cell death autophagy is implied in cancer cells, when the inhibition of mitochondrial ETC induces ROS-dependent autophagic cell death under certain conditions (Frank et al., 2012). In addition, mitochondrial ETC function is reduced under autophagy-deficient conditions. Autophagy-related gene (Atg)-deletion cells were maintained in nonfermentable medium to trigger mitoROS generation

and oxidative phosphorylation. Autophagy inhibition lead to a growth defect with lower oxygen consumption rates and reduction of mitochondrial ETC activities, suggesting autophagy is essential in maintaining the bioreaction of ROS. Although studies have identified that ROS are essential to signal messengers in autophagy induction, the core mechanism of how ROS regulate autophagy remains to be uncovered. However, some modes of ROS-regulated autophagy have been revealed, ROS may regulate autophagy in core mechanisms, transcriptional regulators of autophagic genes, and affiliated or upstream signaling pathways.

ROS-Regulated Core Autophagic Signaling Pathways

Autophagy can be divided into five different stages with distinct core pathways, including induction, nucleation, elongation and completion, docking and fusion, as well as degradation and recycling. During the induction of autophagy, PI3K–Akt–mTORC1 signaling pathway is one of the major mechanisms that controls growth factor response and autophagosome induction. Stimulation of growth factor can lead to the activation of PI3K, thus activating Akt that brings about cell proliferation and the evasion of cell death. mTORC1 is a critical downstream regulator of PI3K–Akt pathway, which can inhibit autophagy as a consequence of the declined activities of ULK1 and mAtg13. ROS have been proved to activate the PI3K–Akt–mTORC1 pathway to affect autophagy. Exogenous hydrogen peroxide treatment activates Akt. In addition, phosphatase and tensin homolog deleted on chromosome ten (PTEN) is the negative regulator of this pathway as well as the ROS targets. Besides the general effect on endogenous ROS, mitoROS specifically oxidize the active site of PTEN on Cys124 and lead to a disulfide formation with Cys71, which results in inactivation of PTEN. In addition to the effect of PTEN, ROS inhibit other phosphatases of Akt, including PP2A and PTP1B. Those phosphatases dephosphorylate and inactivate Akt. Therefore, the inhibitory activity of ROS in PP2A and PTP1B accounts for the activation of PI3K–Akt–mTORC1 signaling pathway and inhibition of autophagy. ROS can inhibit protective autophagy by decreasing the expression of ULK1 in selenite-treated NB4 cells with p-p53, through a phosphorylation event promoted by p70S6K (Ci et al., 2014).

On the other hand, upregulation of ROS toward other factors of PI3K–Akt–mTORC1 pathway can contribute to autophagy induction in an indirect manner, through upregulating AMP-activated protein kinase (AMPK), ROS can induce autophagy. Evidently, oxygen and nutrient deprivation induces AMP/ATP accumulation and triggers mitochondrial oxidative phosphorylation and ROS generation with activation of AMPK. Activated AMPK can dephosphorylate and thus inhibit mTORC1 to autophagy induction. AMPK also phosphorylates ULK1 complex to activate autophagy, which is necessary under starvation conditions. In addition, following H_2O_2 accumulation and oxidative stress, AMPK can be phosphorylated by the upstream kinase AMPK kinase (AMPKK), leading also to the induction of autophagy as well (Li et al., 2013). Inhibition of mTOR activity by ROS activates autophagy, ROS disrupt Akt–mTOR signaling pathway and MMP to induce autophagy, and this inhibition is possible via a BNIP3-dependent pathway, as suggested in C6 glioma cells (Band et al., 2009).

Additionally, Beclin-1 complex has been reported to be upregulated in response to ROS in cancer cells with involvement of Bcl-X_L and Bax. Accumulation of H_2O_2 exerts

cytotoxic effects on malignant glioma U251 cells through H_2O_2-induced autophagy through the Beclin-1 pathways. Similarly, knockdown of Beclin-1 may blockade ROS-induced autophagy triggered by chemical compounds, elucidating the significance of Beclin-1 complex in ROS-induced autophagy (Zhang et al., 2009).

Applying yeast as a model system to characterize the cellular consequence of ATG gene deletions, including those of Atg1, Atg6, Atg8, and Atg12, results showed defects in degradation of mitochondria in stationary culture has been shown. Deletion of Atg5 may also blockade ROS-induced autophagy under photodynamic efficacy in prostate cancer cells. This evidence underlies the participation of two ubiquitin-like conjugation complexes participation in ROS-regulated mechanisms. Core cysteine protease in another ubiquitin-like complex, Atg4, can be regulated by ROS levels to induce autophagy in LC3–PE conjugation. Increased intracellular H_2O_2 under amino acid starvation and inhibits the protease activity of Atg4 via oxidization of a critical cysteine residue near the catalytic site, though it is not clear how inhibition of Atg4 activity activates autophagy. Considering that Atg4 participates in the deconjugation of LC3–PE for recycle use, it is speculated that ROS generation in mitochondria is transient under starvation. It prevents the cleavage of LC3 from the membrane and results in more LC3–PE in cells and leads to more autophagosome formation (Betin et al., 2012). The signaling adaptor p62, which is eliminated through autophagy process, can be accumulated in response to stress accompanied by ROS production. Evidently, p62 upregulation is a mechanism for defective autophagy that contributes directly to tumorigenesis. Deficiency of p62 produces increased ROS levels that account for the enhanced cell death.

ROS-Regulated Other Autophagic Signaling Pathways

ROS can regulate autophagy through transcription factor activity such as NF-κB leading to the induction of autophagy gene expression (Beclin-1 or p62) in cancer cells. In addition, mitogen-activated protein kinases (MAPKs) are downstream effectors of ROS in autophagy induction, as a novel compound was recently found to induce autophagic cell death by stimulating ROS production and activation of ERK and JNK. ROS-induced ATG gene upregulation in skeletal cells requires p38 activation, p38 and p53 also regulate ROS production in turn as positive-feedback responses. One p53-target gene encodes TP53-induced glycolysis and apoptosis regulator (TIGAR), whose inhibition can increase ROS production and enhance ROS-dependent autophagy (Hoshino et al., 2012). The pro-autophagic Ca^{2+} channel IP(3)R can be affected by ROS to increase the intracellular Ca^{2+} level, which has been reported to induce autophagy (Raina et al., 2013) (Figure 12.2).

mitoROS-REGULATED AUTOPHAGIC PATHWAY IN CANCER

Sustaining proliferative signaling, evading growth suppressors, and resisting cell death are all rational complexities of cancers, in which ROS-regulated autophagy plays a dual role in cancer cell death and constitutes multidimensional signaling pathways. Autophagy has two primary and opposing functions (cytoprotective or cytotoxic role) in cancers in response to induced stimuli, such as chemotherapy or radiation. Cytoprotective function can in theory be inhibited for therapeutic advantage by sensitizing the cells to these

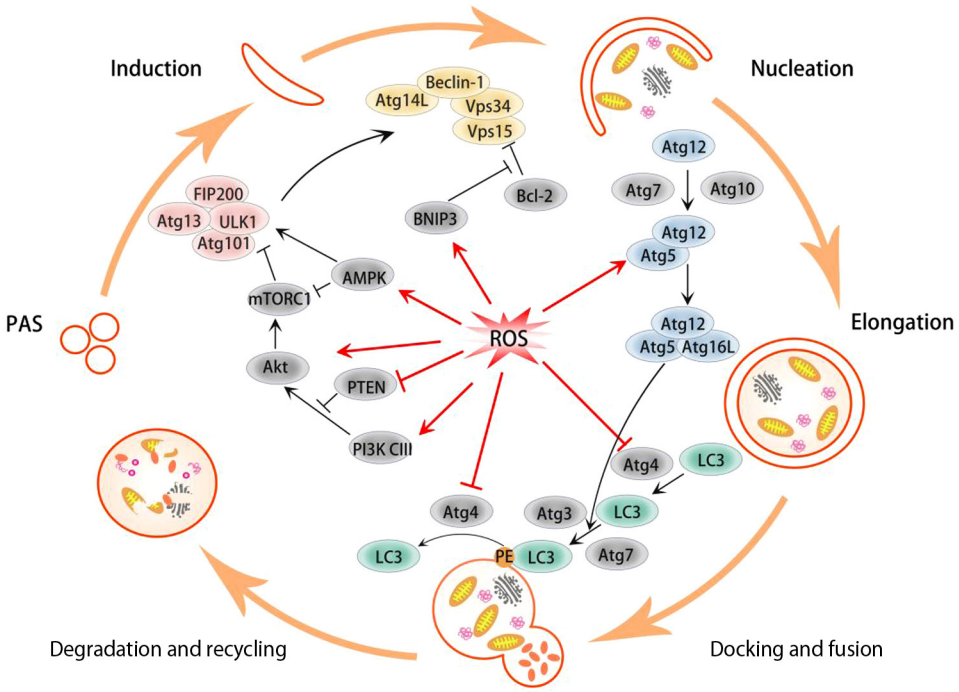


FIGURE 12.2 ROS-regulated autophagic pathways. Autophagy can be divided into five steps: induction, nucleation, elongation, docking and fusion, degradation and recycling. ROS may regulate these five steps through activating or inhibiting various signaling pathways and manipulate autophagy as a survival or programmed cell death role.

treatment modalities, while cytotoxic function to promote cell death can be achieved either alone or in association with apoptosis. Both roles of autophagy have been confirmed in cancer, with large research interests to utilize the switch in cancer therapy (Figure 12.3).

mitoROS-Regulated Autophagy as a Cytoprotective Role in Cancer

As a well-known stress-induced mechanism, autophagy can play a pro-survival role in cancer cells under oxidative stress. Autophagy may degrade damaged organelles via selective autophagy in cancer cells. Chaperone-mediated autophagy is also activated to degrade oxidized proteins damaged by ROS. Under certain conditions, such as treatment with anticancer agents or photodynamic treatment, autophagy can be triggered as a cytoprotective process in various cancer cell lines. For instance, Ciclopirox olamine (CPX, a potential anticancer agent) induces a ROS-dependent autophagy in human rhabdomyosarcoma cells via the activation of JNK signaling pathway. However, autophagy inhibition enhances CPX-induced cell death, indicating that autophagy plays a pro-survival role in human rhabdomyosarcoma cells. Moreover, similar studies about Safingol (an anticancer sphingolipid) in human breast cancer cells and colorectal cancer cells have demonstated the cell repair

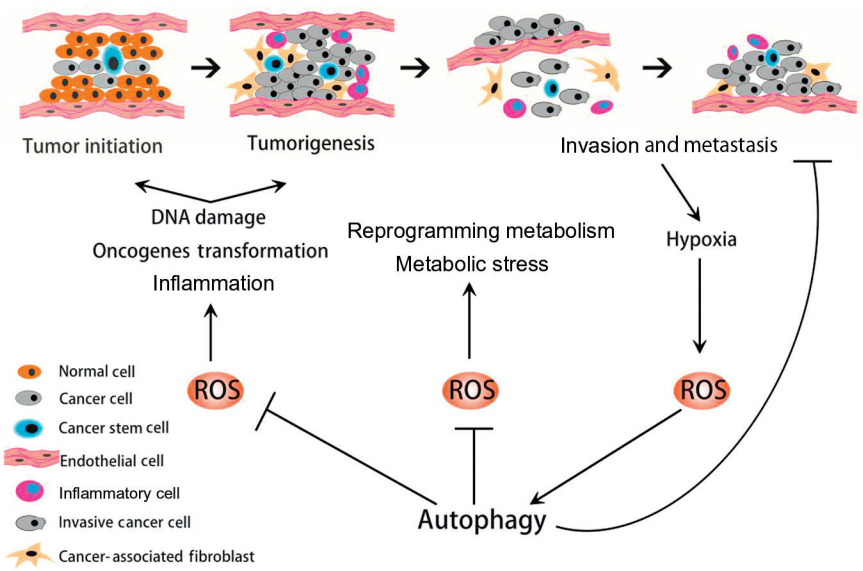


FIGURE 12.3 ROS-regulated autophagy in cancer. A tumor can be recognized as a complex organ and the biology system can be elucidated by studying individual specialized cell types, such as cancer cell, cancer stem cell, inflammatory cell, invasive cancer cell, and cancer-associated fibroblast (CAF). As well as the microenvironment of cancer differentiates during cancer development. ROS-regulated autophagy participates in cancer development with multiple roles. It protects cells from DNA damage and oncogene transformation via removal of ROS. Conversely, it removes ROS thus protects cancer cells against metabolic stress. Interestingly, in cancer environment, CAF may produce signals that are similar to hypoxia, thus triggered autophagic cell death. In addition, ROS-mediated autophagy can also reduce chronic inflammation.

mechanism of autophagy. AMPK, Bcl-xL, and Bax are involved in such regulation of safingol-induced autophagy. Autophagy also protects cancer cells from the accumulation of ROS through mitochondrial dysfunction and involvement of NADPH oxidase in melanoma by proton pump inhibitors, and significantly decreased cytotoxicity of drugs, representing an adaptive survival mechanism to overcome drug-induced cellular stress and cytotoxicity.

mitoROS-Regulated Autophagy as a Programmed Cell Death Role in Cancer

Autophagy caused by massive ROS induction can lead to the excessive self-digestion and eventually cell death. It is demonstrated that triggering autophagic cell death is an attempted target in cancer therapy. Inhibition of the mETC generates ROS and induces autophagic cell death in the various cancer cell lines, including transformed HEK 293, U87, and HeLa. Overexpression of SOD may block such process in HeLa cells decreased autophagy. In addition, inhibition of antioxidant systems, exogenous ROS and metabolic stresses, or anticancer drugs can induce autophagic cell death in a ROS-dependent manner as well. For instance, oxidative stress by exposure to H_2O_2 in C6 glioma cells triggers a BNIP3-dependent autophagic cell death, with dephosphorylation of mTOR and the p70 ribosomal protein S6 kinase (p70S6K). ROS-induced hyperthermic cell death in colon cancer

is detected with autophagic features rather than apoptotic features, suggesting that thermal sensitization via ROS modulation can increase the efficacy of hyperthermia as an anticancer modality. In addition, disruption of cell proliferation regulator may cause ROS-induced autophagic cell death in cancer. Inhibition of gonadotropin-releasing hormone-I (GnRH-I, regulator of prostate cancer cell proliferation) inhibits growth of prostate cancer cells which is associated with decreased MMP and increased levels of ROS. This hormone also induces autophagic cell death accompanied by decreased AKT phosphorylation and increased JNK phosphorylation.

mitoROS-Regulated Autophagy in Cancer Development

Currently, no rationale has been identified for the complex role of autophagy pathway during tumor initiation. Autophagy protects against ROS and therefore inhibits their deleterious effect on DNA mutation. Autophagy also presents a tumor-suppressive role by regulating chronic inflammation caused by ROS. It has been previously shown that elimination of damaged mitochondria by autophagy leads to decreased ROS production. Atg5 or Atg7 deletion can trigger chronic oxidative stress with accumulated mitochondria damage, tissue damage, and inflammation, all favoring tumor initiation (Takamura et al., 2011). Besides accumulation of mitoROS, deletion of Atg16L1, Beclin-1, or LC3B caused autophagy defects confirmed with increased levels of IL-1β and IL-18, leading to an induction of inflammation (Saitoh et al., 2008). Indeed, accumulation of damage mitochondria and oxidative stress suggesting that activation of oncogenic RAS induces autophagy to sustain metabolic needs and lead to an "addiction" of cells to autophagy. Moreover, deficiency in SQSTM1/p62 increases ROS levels following RAS activation, indicating a complexity of ROS regulation mechanisms in cancer initiation (Duran et al., 2008).

Relevant features, including metastasis, epithelial–mesenchymal transition (EMT), and angiogenesis, characterize tumor progression and aggressiveness. Autophagy is activated by hypoxia and ischemia to promote survival of cancer cells. High levels of autophagy are indeed observed in hypoxic regions of tumors. Hypoxia is a prominent feature of tumor cells *in vivo* due to a mismatch between the high proliferative rates of tumor cells and the ability of the blood supply to provide nutrients including oxygen. Hypoxia induces ROS production leading to the stabilization of hypoxia-inducible factor-1α (HIF-1α) (Mazure and Pouyssegur, 2010). HIF-1α, a key regulator of oxygen homeostasis, induces mitophagy through expression of BNIP3 allowing the cells to survive during prolonged hypoxia by preventing increased levels of ROS. Indeed, BNIP3 is an HIF-1α target gene and disrupts the interaction between Beclin-1 and Bcl-2, inducing its release and autophagy induction (Bellot et al., 2009). Disruption of autophagy by Beclin-1, Atg5, or Atg7 knockdown leads to hypoxia-induced cell death. BNIP3-induced autophagy is necessary to prevent the increase in ROS production during hypoxia and therefore represents a survival adaptive mechanism (Mazure and Pouyssegur, 2010). Moreover, autophagy also increases the extracellular release of nuclear protein high-mobility group box protein 1 (HMGB1) depending on its redox status. The secreted HMGB1 can then be recognized by different membrane receptors such as toll-like receptors (TLR2, 4 and 9) or macrophage-1 antigen (Mac-1) leading to the acceleration of the pro-inflammatory response dependent on cytokines and type-I interferon (IFN) and later to tumor progression as well as metastasis (Kang et al., 2013).

High levels of ROS are involved in cancer metastasis. Metastasis is a multistep process that permits migration of cancer cells toward distant organs, for example excessive ROS may accelerate DNA damage in tumor aggressiveness. Notably, ATP production reduction and ROS increase during detachment from the extracellular matrix (ECM) are presented in epithelial cells for metabolism modifications. It caused AMPK-induced autophagy in response to metabolic stress. Also, downstream signals regulated ROS, MAPKs, and p21-activated kinase (PAK), are established to be the major signaling pathways for driving cancer cell metastasis (Buchheit et al., 2012). In the microenvironment of cancer, tumor stroma is characterized by the presence of different stress factors such as hypoxia, high level of ROS, high metabolic stress, and lack of growth factors, including different cell types such as myofibroblasts/cancer-associated fibroblasts (CAFs), immune cells, and endothelial and adipocyte cells. Stroma cells are interacted with tumor cells and can regulate tumor cell development (Zhao et al., 2013). An important discrepancy was recently proposed that CAFs can induce an oxidative stress mimicking hypoxia, increasing ROS production. This stress activates the transcription factors HIF-1α and NF-κB, leading to the induction of autophagy and mitophagy, which affects the microenvironment surrounding the tumor (Martinez-Outschoorn et al., 2010).

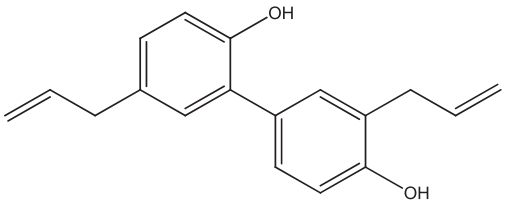
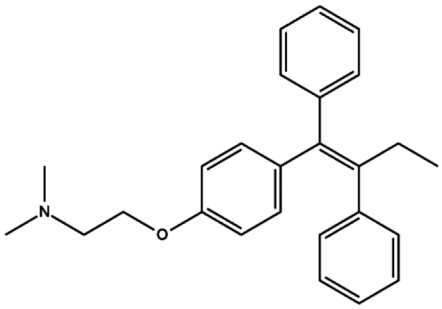
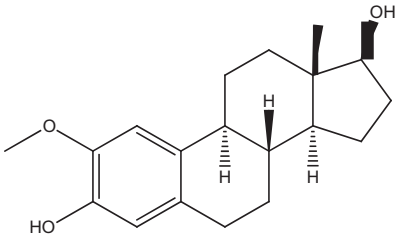
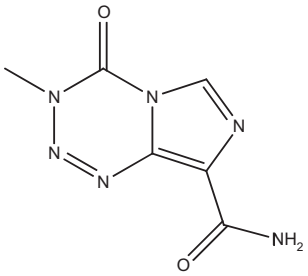
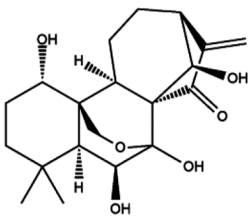
ANTITUMOR DRUGS TARGETING mitoROS-RELATED AUTOPHAGY

Biochemical properties, including the frequently genetic mutations and high oxidative stress, can be exploited in therapeutic strategies, such as pharmacological ROS insults. Nevertheless, to adapt intrinsic oxidative stress, the upregulation of antioxidant capacity constitutes drug resistance in cancer cells; thus, redox modulation to abrogate drug-resistant mechanisms may have significant therapeutic implications (Table 12.1).

One example is the use of autophagy inducer Honokiol in treatment of prostate cancer; however, it is unable to trigger autophagy with treatment of antioxidants (*N*-acetylcysteine, catalase, SOD). The result demonstrates that ROS are critical and direct molecules during pro-autophagy drug treatment (Hahm et al., 2014). Tamoxifen, engaging as an antagonist of mitochondrial ER-β, can downregulate activity of mitochondrial specific SOD, MnSOD, in human breast cancer cells, and thereby increases mitoROS concentrations (Razandi et al., 2013). 2-methoxyestradiol (2-ME) suppresses Akt expression in ROS-mediated (H_2O_2 and $O_2^{-\bullet}$) manner by activating a p53-JNK1 cascade, followed by mTORC1 inhibition and autophagy induction. ROS burst and ERK activation which can be stroked induce cytoprotective autophagy by both Temozolomide and valproic in malignant glioma (Trachootham et al., 2009).

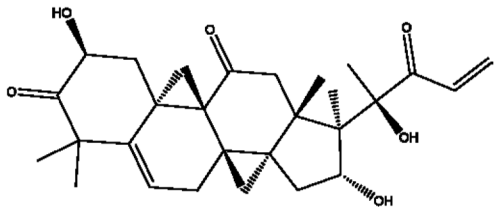
Studies of how Oridonin contributes to ROS-mediated autophagy have been conducted in various cancers, including human cervical carcinoma, lymphoma, and epidermoid carcinoma. Oridonin is an active diterpenoid isolated from *Rabdosia rubescen* and shows high activity to induce the generation of ROS, such as H_2O_2 and OH. Oridonin enhances phagocytosis of apoptotic cells by increasing the phosphorylation of Bcl-2, meanwhile, it can trigger autophgic cell death. In human lymphoma U937 cells, treatment with oridonin showed that H_2O_2 and OH were positive regulators of cell death by downregulating MMP and inducing

TABLE 12.1 Anticancer Drug Implications in ROS-Regulated Autophagy

Drugs	Cancer Types	Structure	Autophagy-Modulating Mechanism
Honokiol	Human prostate cancers		Induce ROS-dependent autophagy cytoprotectively
Tamoxifen	Human breast cancer		Down-regulate activity of MnSOD to increase mitoROS
2-Methoxyestradiol	Mouse glioma		Inducing dephosphorylation of mTORC1 and $_{p70}^{S6K}$
Temozolomide			
Oridonin	Human lymphoma Human epidermoid carcino ma		Down-regulate MMP to induce autophagy Activate PI3K–Akt and PLCγ–Ras–Raf–ERK signaling pathways

(Continued)

TABLE 12.1 (Continued)

Drugs	Cancer Types	Structure	Autophagy-Modulating Mechanism
Cucurbitacin	Human cervical cancer		Activate ERK and NK signaling pathway via ROS generation

autophagy (Wen et al., 2013). H_2O_2 and OH generation also activates PI3K–Akt and phospholipase C gamma-protein kinase C (PLC gamma)–Ras–Raf–ERK signaling pathways, which are suppressed by ROS scavenger *N*-acetyl-L-cysteine, H_2O_2 scavenger catalase, or OH scavenger GSH. However, superoxide anion ($O_2^{-\bullet}$) and its scavenger SOD did not significantly affect these oridonin-induced biological processes, and the same conclusion was observed in epidermoid carcinoma A431 cells (Zang et al., 2013; Yu et al., 2012).

Apoptosis and autophagy seem to be interconnected positively or negatively in certain circumstances, referring to the "molecular switches" between them. Our laboratory has found a mannose-binding lectin, *Polygonatum cyrtonema* lectin, can induce autophagy and apoptosis in human melanoma A375 cells through mitochondria-mediated ROS–p38–p53 pathway. PCL regulates Bax, Bcl-xL, and Bcl-2 proteins, leading to mitochondrial depolarization, cyto c release, and caspase activation. Meanwhile, PCL treatment also abrogates glutathione antioxidant system and induces mitochondria to generate ROS accumulation, which results in p38–p53 activation (Liu et al., 2009). During the switch between autophagy and apoptosis, the molecular mechanisms underlying cucurbitacin-induced cell death are different from the PCL, particularly in the involvement of autophagy. Cucurbitacin is a small molecule of STAT3 inhibitor with enhanced apoptosis and autophagy. It promotes excessive conversion of LC3-II and accumulation of autophagosomes independent of its ability to inhibit STAT3 function, but mainly mediated by enhanced production of mitoROS. ROS subsequently activates ERK and JNK, providing new insights that support cucurbitacin as an anticancer drug via the modulation of the balance between autophagy and apoptosis (Zhang et al., 2012a,b).

CONCLUSIONS AND PERSPECTIVES

How the dual role of autophagy participate in cancer development has puzzled researchers for years; and besides cytotoxic and cytoprotective roles, recently two new functional roles of autophagy (cytostatic and nonprotective) have been proposed (Gewirtz, 2014). The complexity of autophagy seems to aggravate with consideration of ROS have dual roles in metabolism as well. Energy metabolism dysfunctions of cancer cells are partly contributed by the chronic and uncontrolled cell proliferation. One of the characteristics of neoplastic

disease is adjustments of energy metabolism, which is applied in order to fuel cell growth and division, such as aerobic disorders. Cancer cells, under anaerobic conditions, have glycolysis that favored relatively little pyruvate to dispatch the oxygen-consuming mitochondria (Hanahan and Weinberg, 2011). Therefore, deconvolution of relations of mitoROS and autophagy is important.

However, limitations of current studies are concentrating on the mechanism of how specific ROS is participate in cellular mechanism, though many studies use H_2O_2 as an ROS source and H_2O_2 is a relatively stable signaling molecule in many signal-transduction pathways, it is now thought that $O_2^{-\bullet}$, rather than H_2O_2, is the major ROS that regulates starvation- and mitochondria ETC inhibition-induced autophagy (Chen et al., 2009). Thus, applying current techniques to distinguish and measure which type of ROS are participating in autophagy regulation in cancers is as important as the complex redox-regulation systems in cancer.

Cancer cells contain a repertoire of replicative immortality, cell death evasion, proliferative signaling sustention, and other biological capabilities, which orchestrate complex mechanisms in response against therapy. The double-edge role of autophagy in cancer has lead researchers to develop novel strategies in cancer treatment, such as clinical trial of hydroxychloroquine with DNA damage therapy. Recognition of these widespread regulation mechanisms of ROS-mediated autophagy in cancer development will increasingly affect the progress of new strategies to treat human cancer.

References

Band, M., Joel, A., Hernandez, A., et al., 2009. Hypoxia-induced BNIP3 expression and mitophagy: *in vivo* comparison of the rat and the hypoxia-tolerant mole rat, *Spalax ehrenbergi*. FASEB J. 23, 2327–2335.

Bellot, G., Garcia-Medina, R., Gounon, P., et al., 2009. Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains. Mol. Cell Biol. 29, 2570–2581.

Betin, V.M.S., MacVicar, T.D.B., Parsons, S.F., et al., 2012. A cryptic mitochondrial targeting motif in Atg4D links caspase cleavage with mitochondrial import and oxidative stress. Autophagy 8, 664–676.

Bhogal, R.H., Weston, C.J., Curbishley, S.M., et al., 2012. Autophagy: a cyto-protective mechanism which prevents primary human hepatocyte apoptosis during oxidative stress. Autophagy 8, 545–558.

Buchheit, C.L., Rayavarapu, R.R., Schafer, Z.T., 2012. The regulation of cancer cell death and metabolism by extracellular matrix attachment. Semin. Cell Dev. Biol. 23, 402–411.

Chen, Y., Azad, M.B., Gibson, S.B., 2009. Superoxide is the major reactive oxygen species regulating autophagy. Cell Death Differ. 16, 1040–1052.

Chourasia, A.H., Boland, M.L., Macleod, K.F., 2015. Mitophagy and cancer. Cancer Metab. 3, 4.

Ci, Y., Shi, K., An, J., et al., 2014. ROS inhibit autophagy by downregulating ULK1 mediated by the phosphorylation of p53 in selenite-treated NB4 cells. Cell Death Dis. 5, e1542.

Duran, A., Linares, J.F., Galvez, A.S., et al., 2008. The signaling adaptor p62 is an important NF-kappaB mediator in tumorigenesis. Cancer Cell 13, 343–354.

Frank, M., Duvezin-Caubet, S., Koob, S., et al., 2012. Mitophagy is triggered by mild oxidative stress in a mitochondrial fission dependent manner. Biochim. Biophys. Acta 1823, 2297–2310.

Gewirtz, D.A., 2014. The four faces of autophagy: implications for cancer therapy. Cancer Res. 74, 647–651.

Hahm, E.R., Sakao, K., Singh, S.V., 2014. Honokiol activates reactive oxygen species-mediated cytoprotective autophagy in human prostate cancer cells. Prostate 74, 1209–1221.

Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. Cell 144, 646–674.

Hoshino, A., Matoba, S., Iwai-Kanai, E., et al., 2012. p53-TIGAR axis attenuates mitophagy to exacerbate cardiac damage after ischemia. J. Mol. Cell. Cardiol. 52, 175–184.

Kang, R., Zhang, Q., Zeh, H.J., et al., 2013. HMGB1 in cancer: good, bad, or both? Clin. Cancer Res. 19, 4046–4057.
Li, L., Chen, Y., Gibson, S.B., 2013. Starvation-induced autophagy is regulated by mitochondrial reactive oxygen species leading to AMPK activation. Cell. Signal. 25, 50–65.
Li, Z., Yang, Y., Ming, M., et al., 2011. Mitochondrial ROS generation for regulation of autophagic pathways in cancer. Biochem. Biophys. Res. Commun. 414, 5–8.
Liu, B., Cheng, Y., Bian, H.J., et al., 2009. Molecular mechanisms of *Polygonatum cyrtonema* lectin-induced apoptosis and autophagy in cancer cells. Autophagy 5, 253–255.
Martinez-Outschoorn, U.E., Trimmer, C., Lin, Z., et al., 2010. Autophagy in cancer associated fibroblasts promotes tumor cell survival: role of hypoxia, HIF1 induction and NF-κB activation in the tumor stromal microenvironment. Cell Cycle 9, 3515–3533.
Mazure, N.M., Pouyssegur, J., 2010. Hypoxia-induced autophagy: cell death or cell survival? Curr. Opin. Cell Biol. 22, 177–180.
Murphy, M.P., 2009. How mitochondria produce reactive oxygen species. Biochem. J. 417, 1–13.
Müller, M., Lu, K., Reichert, A.S., 2015. Mitophagy and mitochondrial dynamics in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta. http://dx.doi.org/10.1016/j.bbamcr.2015.02.024.
Raina, K., Agarwal, C., Wadhwa, R., et al., 2013. Energy deprivation by silibinin in colorectal cancer cells: a double-edged sword targeting both apoptotic and autophagic machineries. Autophagy 9, 697–713.
Razandi, M., Pedram, A., Jordan, V.C., et al., 2013. Tamoxifen regulates cell fate through mitochondrial estrogen receptor beta in breast cancer. Oncogene 32, 3274–3285.
Saitoh, T., Fujita, N., Jang, M.H., et al., 2008. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. Nature 456, 264–268.
Takamura, A., Komatsu, M., Hara, T., et al., 2011. Autophagy-deficient mice develop multiple liver tumors. Genes Dev. 25, 795–800.
Trachootham, D., Alexandre, J., Huang, P., 2009. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat. Rev. Drug Discov. 8, 579–591.
Venditti, P., Di Stefano, L., Di Meo, S., 2013. Mitochondrial metabolism of reactive oxygen species. Mitochondrion 13, 71–82.
Wen, X., Wu, J., Wang, F., et al., 2013. Deconvoluting the role of reactive oxygen species and autophagy in human diseases. Free Radic. Biol. Med. 65, 402–410.
Yu, Y., Fan, S.M., Song, J.K., et al., 2012. Hydroxyl radical (.OH) played a pivotal role in oridonin-induced apoptosis and autophagy in human epidermoid carcinoma A431 cells. Biol. Pharm. Bull. 35, 2148–2159.
Zang, L., He, H., Xu, Q., et al., 2013. Reactive oxygen species H2O2 and *OH, but not O2*(-) promote oridonin-induced phagocytosis of apoptotic cells by human histocytic lymphoma U937 cells. Int. Immunopharmacol. 15, 414–423.
Zhang, H., Kong, X., Kang, J., et al., 2009. Oxidative stress induces parallel autophagy and mitochondria dysfunction in human glioma U251 cells. Toxicol. Sci. 110, 376–388.
Zhang, T., Li, Y., Park, K.A., et al., 2012a. Cucurbitacin induces autophagy through mitochondrial ROS production which counteracts to limit caspase-dependent apoptosis. Autophagy 8, 559–576.
Zhang, T., Qi, Y., Liao, M., et al., 2012b. Autophagy is a cell self-protective mechanism against arsenic-induced cell transformation. Toxicol. Sci. 130, 298–308.
Zhao, X., He, Y., Chen, H., 2013. Autophagic tumor stroma: mechanisms and roles in tumor growth and progression. Int. J. Cancer 132, 1–8.

CHAPTER

13

Role of Autophagy in Cancer Therapy

Yuting Ma

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Abstract

Autophagy is an adaptive response for cells to cope with endogenous or exogenous stress under unfavorable microenvironments or infectious insult. A large group of autophagy-related proteins coordinate to sequester cytosolic cargos or invading microorganisms in double-membrane vesicles called autophagosomes, which then fuse with lysosomes for their degradation. During the process of oncogenesis, autophagy maintains genome stability, eliminates abnormal organelles or protein aggregation to prevent malignant transformation. However, it also reinforces tumor cell adaptation to untoward microenvironment and facilitates tumor progression. Upon chemotherapy or radiotherapy treatment, autophagy antagonizes, cooperates, or accompanies diverse cell death pathways. Therefore, tumor cells could either utilize autophagy as a pro-survival mechanism or end up with autophagy-related cell demise. Autophagy in tumor cells modulates their immunogenicity, whereas autophagy taking place in immune cells affects their differentiation, survival,

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migration, and function. Reciprocally, immune effectors could regulate autophagy process. Pharmacological manipulation of autophagy is under clinical investigations, either used as a monotherapy or in combination with chemotherapies, radiotherapies, or immunotherapies (such as vaccination). However, major efforts should be made to clarify under which circumstances and to which extent inducers and inhibitors of autophagy are beneficial in clinical practice.

INTRODUCTION

Autophagy (from the Greek, "self-eating") is an evolutionarily-conserved cellular response to maintain homeostasis. It occurs either in physiological or pathological condition as a housekeeping or stress response to adverse extracellular or intracellular triggers, such as nutrient starvation, growth factor deprivation, hypoxia, infection, protein aggregation, or mitochondrial damage (Murrow and Debnath, 2013). This catabolic process is generally regarded as "cytoprotective" due to its capability of satisfying urgent metabolic needs through "self-consumption" and preventing the accumulation of damaged organelles and misfolded proteins.

The identification of autophagy-related genes in the yeast and their homologs in other eukaryotic organisms fueled our understanding of this process. Autophagy requires coordinated activation of multiple molecules in a series of sequential steps, including: (1) ULK1 (UNC-51-like kinase 1) and mTORC1 (mTOR complex 1, a negative regulator of autophagy) for autophagy induction (Kim et al., 2011a); (2) BECLIN1-VPS34 (lipid kinase vacuolar protein sorting 34) core complex for the nucleation of the isolation membrane (Kang et al., 2011), ATG9 and VMP1 (vacuole membrane protein 1) for the further recruitment of lipids to the isolation membrane (Molejon et al., 2013; Reggiori and Klionsky, 2006); (3) two UBL (ubiquitin-like) conjugation systems operating on ATG12 and LC3 (microtubule-associated protein 1 light chain 3) for autophagosome formation (Ohsumi, 2001); (4) a series of adaptors, such as SQSTM1 (sequestosome 1, p62), which specifically interact with LC3-like proteins and recognize Lys63-linked ubiquitin chains-tagged autophagic cargos for their sequestration by autophagosomes (Johansen and Lamark, 2011); (5) several SNARE-like proteins mediated fusion between autophagosomes and lysosomes (Tumbarello et al., 2012); (6) various lysosomal hydrolases, which catalyze proteins, lipids, and nucleic acids in the acidic autolysosomes (Kroemer and Jaattela, 2005).

Besides the discovery of molecular mechanism of autophagy, this process is proved to participate in various pathological changes, including cancer, neurodegenerative diseases, infection, metabolic diseases, and aging (Choi et al., 2013). The essential autophagy regulator BECLIN1 provided the first link between autophagy and cancer. BECLIN1 was reported allelically deleted in several breast and ovarian cancers and its monoallelic loss resulted in spontaneous tumorigenesis (Qu et al., 2003). Consistently, BECLIN1 overexpression promoted autophagy as well as inhibited tumorigenesis in immune-deficient mice. These pioneered studies suggest that autophagy could act as an intrinsic tumor-suppressive mechanism.

Accumulating data suggest that autophagy accompanies each phase of oncogenesis. During tumor initiation, autophagy exerts an important role in restraining genetic instability, preventing protein misfolding/aggregation and mitochondria damage (Mathew et al., 2007,

2009), as well as promoting immunosurveillance (Rao et al., 2014). During tumor progression, rapid proliferation of neoplastic cells augments their metabolic demands, while abnormal tumor vasculature limits their access to oxygen, nutrients, and growth factors. Cancer cells are frequently observed undergoing metabolic adaptations characterized as elevated aerobic glycolytic and higher reactive oxygen species (ROS) generation, resulting in L-lactate accumulation and acidic extracellular pH (Warburg effect), as well as redox imbalance (Hanahan and Weinburg, 2011). Additionally, host immune system creates the pressure for neoplastic lesions destruction (a process called "immunoediting") (Schreiber et al., 2011). Thus, malignant cells need to overcome untoward microenvironment through autophagy-mediated cytoprotection. Furthermore, cancer stem cells, which often locate at the hypoxic core of solid tumors and are sensitive to oxidative stress, rely on autophagy to maintain their survival and self-renewal (Vessoni et al., 2012). During the phase of tumor metastasis, autophagy may limit tumor dissemination by restricting the outgrowth of dormant cells into macrometastases, or by reducing tumor cell necrosis and tumor-promoting inflammation (Degenhardt et al., 2006), while empowering antitumor immune responses. However, autophagy may also facilitate tumor adaptation to nonhostile environmental stresses, such as anoikis (a form of cell death induced by detachment from extracellular matrix (ECM)) (Debnath, 2008). The contribution of autophagy during therapeutic intervention is also paradoxical. It could either render cytotoxicity resistance or trigger autophagy-related cell death.

These exciting discoveries raise the question that whether autophagy could be manipulated to tackle cancer. Experimental models and clinical trials targeting autophagy in cancer prevention and therapy are under investigation. Although important strides have been made, several key issues remain to be addressed. Whether autophagy should be enhanced or blocked is still under debate. Biomarkers to measure autophagy modulation during treatment are missing. Whether the beneficial effect of autophagy-targeting drugs indeed act through autophagy modulation. How to minimize the side effects on normal tissues upon autophagy induction/inhibition? A comprehensive understanding of autophagy in cancer is important for its optimal exploitation for therapeutic advantages.

DUAL ROLE OF AUTOPHAGY IN CANCER

Tumor-Limiting Effect of Autophagy

Reduced autophagy was firstly linked to better survival and outgrowth of pre-neoplastic cells in a carcinogen-induced rat hepatocarcinoma model, suggesting the tumor-suppressive property of autophagy (Schwarze and Seglen, 1985; Seglen et al., 1986). Also, tumor cells and their precursors may produce autophagy-suppressive factors in an autocrine or paracrine mode (Kisen et al., 1993). After the identification of autophagy-related genes, accumulating data confirmed the link between autophagy-deficiency and a tumor-prone phenotype. Autophagy-deficient murine cells (B*eclin*$^{+/-}$, *Atg5*$^{-/-}$), which fail to adapt to metabolic stress, have evident DNA damage, gene amplification, increased frequency of chromosome gains/losses, and aneuploidy (Mathew et al., 2007). Also, these cells contain elevated ER chaperones, protein aggregates, protein disulphide isomerases (indicating protein quality control failure), and damaged mitochondria (a potential source of ROS). Elevated oxidative

stress and DNA damage response attribute directly to the persistence of p62 in autophagy-deficient cells, which accelerates aneuploidy progression and proliferation of hepatocellular carcinoma (HCC) through altering the canonical and noncanonical arms of NF-kB pathway (Mathew et al., 2009). Sequestosome 1/p62 is a major constituent of intracytoplasmic hyaline bodies in human HCC but undetectable in nonneoplastic liver tissue (Stumptner et al., 1999). Overabundance of p62 is also observed in human breast cancers relative to normal breast tissues and significantly correlates with HER2 overexpression (Rolland et al., 2007). Heterozygous disruption of BECLIN1 compromises autophagy activation, increases cellular proliferation and spontaneous tumor formation in mice. Also, BECLIN1 is monoallelically deleted in 40–75% of human sporadic breast, ovarian, and prostate cancer cases, at least partially due to defective autophagy and plays a causal role in cancer (Qu et al., 2003). Targeted disruption of the *Atg4c*, an essential component of the Atg8/LC3 UBL conjugation system, increases susceptibility of mice to carcinogen-induced fibrosarcomas (Marino et al., 2007). Complete loss of BIF-1, which interacts with BECLIN1 through ultraviolet irradiation resistance associated gene and functions as a positive regulator of autophagy, significantly enhances spontaneous tumor development in mice (Takahashi et al., 2007). Both systemic mosaic *Atg5* deletion and liver-specific *Atg7* deletion induce benign liver adenomas from autophagy-deficient hepatocytes (Takamura et al., 2011). Thus, autophagy acts as an essential cell-autonomous homeostatic mechanism to limit tumor initiation and progression.

Cellular senescence represents a state of permanent cell-cycle exit in metabolically viable cells marked by the expression of cell cycle inhibitors. Senescence could be induced by telomere erosion, oncogene expression, oxidative stress, and cytotoxic therapies (Bartkova et al., 2006; Ewald et al., 2010; Singh et al., 2012). Autophagy and senescence are usually not considered as two inexorably linked cellular responses to stress. However, autophagy was shown to mediate *Ras* oncogene-induced senescence (Young et al., 2009). Overexpressing autophagy genes (e.g., *ULK3, BNIP3, CTSB, or ATG16L1*) induces a constitutive autophagic phenotype and features of senescence in human fibroblasts and breast cancer cells (Capparelli et al., 2012; Young et al., 2009), while inhibiting autophagy delays oncogene- or chemotherapy-induced senescence execution (Goehe et al., 2012; Young et al., 2009). Senescence is also involved in autophagy-mediated tumor dormancy (Gewirtz, 2009). Interestingly, senescence triggers the expression of chemokines and cytokines which either reinforce growth arrest through a self-amplifying secretory network (e.g., CXCR2 and its ligands) (Acosta et al., 2008) or attract the immune effector cells (Xue et al., 2007) for the clearance of senescent cells. Thus, autophagy can control oncogenesis by facilitating senescence.

Inflammation promotes tumor initiation and promotion (Grivennikov et al., 2010). Autophagy may also protect against tumorigenesis by mitigating metabolic stress and, in concert with apoptosis, by preventing necrotic cell death and chronic inflammation (Degenhardt et al., 2006). Oxidative stress could trigger cytosolic translocation of chromatin-associated nuclear protein high-mobility group box 1 (HMGB1), an extracellular damage-associated molecular pattern molecule (DAMP), which directly interacts with the BECLIN1 displacing Bcl-2 to induce autophagy and prevent cell death and inflammation (Tang et al., 2010).

In summary, the homeostatic function of autophagy, especially at precancerous stages, is dominant to prevent normal cells from malignant transformation by relieving stimuli stress, eliminating damaged organelles, reinstating redox state, and reducing genomic instability. Autophagy also promotes oncogene-induced senescence as a barrier to carcinogenesis.

Meanwhile, it limits necrosis and inflammation persistence, both of which favor primary tumor outgrowth.

Tumor-Promoting Effect of Autophagy

Emerging evidence indicates that cytoprotective function of autophagy could serve to maintain tumor cell survival. Autophagy can be induced by extrinsic factors and intrinsic triggers, such as metabolic stress, hypoxia, acidic pH, activation of oncogenes, or tumor-suppressor genes. It was observed that significantly higher degree of autophagy existed in the tumor center rather than tumor margins (Fang et al., 2013), contributing to the survival of interior neoplastic cells under hypoxia and limited access to nutrients or growth factors. The occurrence of autophagy (marked by punctate staining pattern of LC3B) strongly associated with hypoxic tumor regions (evaluated by positive pimonidazole staining) distal to blood vessels.

Hypoxic pressure could induce autophagy-related gene expression (*ATG5, LC3B, BNIP3*) through a unfolded protein response- or hypoxia-inducible factor-1α (HIF-1α) dependent pathway (Hu et al., 2012; Rouschop et al., 2010), leading to malignant cells survival and treatment resistance. Upregulation of BNIP3 by hypoxia facilitates mitophagy induction which removes the trigger of cell death (ROS) originated from the mitochondria. Clinical observation confirmed the correlation of immunostaining for BNIP3 with poor survival in non-small-cell lung carcinomas (NSCLCs) (Giatromanolaki et al., 2004) and endometrial cancers (Giatromanolaki et al., 2008). Tumor cells exposed to low pH upregulate *ATG5* and *BNIP3* expression and increase their LC3-positive punctate vesicles content. These elevated autophagy markers could be maintained chronically (several months), rendering survival adaptation of tumor cells to acidic tumor microenvironment (Wojtkowiak et al., 2012). High expression of autophagy marker LC3B (and its distribution) was reported to correlate with poor prognosis in various tumor types (Fujii et al., 2008; Han et al., 2011).

Oncogenes and tumor-suppressor genes can be either positively or negatively linked to autophagy induction with relevance to tumor progression. Small GTPase Ras is an oncogene involved in the regulation of various cell-signaling pathways, which is mutated in 33% of human cancers (Karnoub and Weinburg, 2008). Ras could positively regulate the class I PI3K/AKT/mTOR1 pathway and act as a negative regulator of autophagy (Schmukler et al., 2014). Conversely, Ras can promote autophagy via the Rac1/MKK7/JNK pathway and subsequent upregulation of ATG5 (Byun et al., 2009), or through the Raf-1/MEK1/2/ERK pathway which inhibits the binding of Bcl-2/Mcl-1 to BECLIN1 (Wu et al., 2011). Human cancer cell lines bearing activating mutations in H-ras or K-ras show high basal autophagy and they rely on autophagy to maintain functional metabolism and mitochondrial integrity, facilitating tumor cell survival and tumorogenesis (Guo et al., 2011). Increased LC3II were observed in HCC tissues with low glucose uptake and high K-Ras expression (Kim et al., 2011b). Autophagy is a critical mediator of Ras-induced senescence for tumor inhibition (Young et al., 2009), whereas it was also reported that overexpressing autophagy gene Atg5 could bypass Ras-induced senescence (Wang et al., 2012).

Tumor-suppressor gene p53, the "guardian of the cellular genome," could also modulate autophagy induction. Cancer cells could hijack the wild-type p53 which posttranscriptionally down-regulates LC3 to enable reduced, yet sustainable autophagic flux. This strategy increased their fitness under limited nutrient supply (Scherz-Shouval et al., 2010). In

p53-proficient cells, p53 activates in response to cellular stress. Several autophagy-related genes, such as β1 and β2 subunits of the AMPK, TSC2, PTEN, can be trans-activated by p53 subsequently, permitting the activation of AMPK and TSC1/TSC2 for the negative regulating mTOR activity (Feng et al., 2007). The pro-tumoral role of autophagy may depend on intact p53 expression. In a humanized mouse bearing an activated oncogenic allele of Kras in pancreases, autophagy deficiency (*Atg7* or *Atg5* deletion with Cre-loxP technology) causes the accumulation of low-grade, premalignant pancreatic intraepithelial neoplasia lesions, with limited progression to high-grade pancreatic intraepithelial neoplasias and pancreatic ductal adenocarcinoma. In sharp contrast, autophagy deficiency no longer blocks tumor progression, but accelerates tumor onset instead, when the mice harbors p53 deficiency in the meantime. This is due to reduced autophagy flux in the absence of p53, even if autophagy machineries are normal. Combined loss of *p53* and *Atg7* triggers metabolic changes to fuel tumor growth (Rosenfeldt et al., 2013). Therefore, p53 could function as a pro-autophagic factor to confer stress tolerance. In contrast, the functional absence of p53, by deletion, depletion, or pharmaceutical inhibition, also leads to autophagy induction through activating AMPK and inhibiting mTOR. Enhanced autophagy improves the survival of p53-deficient cancer cells under conditions of hypoxia and nutrient deprivation. Interestingly, overexpressing cytoplasmic-located p53 was able to repress the enhanced autophagy in p53-null cells, whereas introducing nuclear-locked p53 (harboring a disrupted nuclear export signal) failed to do so. Nonetheless, autophagy-inducing stimuli cause concomitant depletion of p53 in the cytoplasm (as well as in the nuclei), which in turn unleashed autophagy inhibition by p53 (Tasdemir et al., 2008). Mutations in p53 occur almost 50% of all human tumors (Soussi and Wiman, 2007). It remains plausible that diverse p53 mutations may regulate tumor progression through autophagy differently.

Collectively, autophagy could fuel tumor progression in the sense that it functions as a pressure-coping mechanism in response to intrinsic and extrinsic factors.

THERAPY-INDUCED STRESS AND AUTOPHAGY

Current cancer therapies including ionizing radiation and anticancer drugs have been shown to affect autophagy (Levy and Thorburn, 2011). Radiotherapy causes cell cycle arrest, senescence, or death directly through inducing DNA damage, misrepair and chromosome aberrations, or indirectly by creating free radicals that trigger oxidative stress. Various families of chemotherapeutic drugs (including alkylating agents, DNA damaging agents, histone deacetylase inhibitors, kinase inhibitors, antimetabolites, death receptor agonists, hormonal agents, antiangiogenic agents, and proteasome inhibitors) have been developed to deliver lethal stress preferentially to cancer cells. Autophagy is often induced in chemotherapy- or radiation-treated tumor cells (Kondo and Kondo, 2006). But the role of autophagy in radiotherapy and chemotherapy remains controversial.

Cytoprotective and Cytotoxic Role of Therapy-Induced Autophagy

On the one hand, autophagy confers tumor cells superior tolerance to diverse stresses, therefore limiting damage, improving viability, and facilitating therapeutic resistance.

Consistent with this scenario, blocking autophagy with genetic approaches or pharmacological inhibitors should abolish its cytoprotective function, enhance the efficacy of anticancer drugs, and prevent cancer recurrence.

It was reported that γ-irradiation induced augmented mRNA transcription of autophagy-related genes and autophagosome accumulation in various human cancer cell lines. Targeted knockdown of these autophagy-related genes or pretreatment with autophagy inhibitors (3-methyladenine (3-MA) or chloroquine) reduced their clonogenic survival and sensitized resistant carcinoma cells to radiation (Apel et al., 2008; Chaachouay et al., 2011). Autophagy also acts as a self-protective mechanism against chemotherapy-induced tumor elimination (Amaravadi et al., 2007). CD133 is recognized as a cancer stem cell marker. $CD133^+$ cells from glioma, NSCLC, and HCC showed greater radiochemoresistance and tumorigenicity, due to a higher degree of autophagy, cell cycle arrest, apoptosis resistance, and lower ROS generation. Silencing autophagy-related genes, pretreatment with autophagy inhibitor bafilomycin A1 (BafA1) (which inhibits the acidification of lysosomes and autolysosome formation) or STAT3 inhibitor cucurbitacin I abolished the stem cell–like properties and enhanced the radiochemosensitivity of $CD133^+$ tumor cells (Lomonaco et al., 2009).

One the other hand, autophagy contributes to cell death and it is indispensable for optimal therapeutic effect. Autophagy-induced cell death may result from excessive self-cannibalism which no longer supports cell survival under prolonged stress. Indeed, autophagy accompanying nonapoptotic cancer cell death has been described (Kanzawa et al., 2005). Fukumoto's group established some radioresistant human liver and oral cancer cell lines by daily exposure of tumor cells to low dose of irradiation. Compared with their parental cell lines, these cells showed similar apoptosis but prominent reduction in autophagy upon X-ray irradiation. Inhibition of mTORC1 with rapamycin facilitated their autophagy induction and abrogated their radioresistant phenotype. Consistently, 3-MA treatment or BECLIN1 knockdown made their parental cells radioresistant (Kuwahara et al., 2011). A novel small molecule (STF-62247) was reported to promote cell death through induction of autophagy in *von Hippel-Lindau*-deficient renal cell carcinoma cells (Turcotte et al., 2008). Interestingly, vitamin D analog (1,25-dihydroxyvitamin D_3) could switch off cytoprotective function of autophagy in irradiated breast tumor cells and confer them sensitive to irradiation (Bristol et al., 2012).

Cross Talk Between Autophagy and Cell Death Modalities

Autophagic vacuolization is often observed preceding or accompanying cell death, leading to a commonly used nomenclature "autophagic cell death." However, it is still ambiguous whether autophagy can function as a primary death modality *per se*. Shen and colleagues performed a high-content screen on the induction of autophagy, apoptosis, and necrosis with a National Cancer Institute (NCI) panel containing around 1400 cytotoxic agents. Many of these drugs could induce a "pure" autophagic phenotype and *bona fide* autophagic flux in a reporter osteosarcoma cell line. Surprisingly, none of these drugs could induce cell death by autophagy (Shen et al., 2011).

Autophagy usually acts as a pro-survival mechanism to relieve lethal stress and a barrier to cell death execution. Abundance of evidence suggested that autophagy antagonizes apoptotic pathway (Boya et al., 2005). Once stressors reach a critical point and this

barrier has been overcome, autophagy is hypothesized to revert and mediate cell death, either participating in or contributing to apoptotic cell death (Nikoletopoulou et al., 2013). It is exemplified by pan-Bcl-2 inhibitor treated glioblastoma cells which exhibited increased autophagy, while knockdown *Beclin 1* or *Atg5* expression increased cell survival (Voss et al., 2010). Autophagy could also collaborate with apoptosis. Forced expression of *Atg5* provoked apoptotic cell death and sensitized tumor cells to anticancer treatment both *in vitro* and *in vivo*, while silencing *Atg5* resulted in partial chemoresistance. This was due to calpain-mediated ATG5 cleavage resulting in the translocation of truncated ATG5 to mitochondria to initiate the intrinsic apoptotic pathway (Yousefi et al., 2006). Disabled apoptosis frequently occurs in human cancers while autophagy could execute cell death in apoptosis-resistant cells or when apoptosis pathway is blocked (Vandenabeele et al., 2006). In apoptosis-deficient cancer cells, autophagy acts as a prominent cause of cell death and increases radiosensitivity of neoplastic cells (Zhuang et al., 2011).

Necrosis was regarded as an uncontrolled accidental process while recent studies showed that it could be a highly coordinated program. Although unique markers for necrosis have not been identified, important progresses have been achieved in the mechanistic study of regulated necrosis (necroptosis). Necroptosis can be triggered by tumor necrosis factor (TNF) family members, Toll-like receptors (TLR3 and TLR4), and DNA/RNA sensors (DAI and possibly RIG-1 and MDA5). The execution of necroptosis depends on the formation of necrosome composed of CYLD (cylindromatosis), RIPK1 (receptor-interacting serine/threonine-protein kinase 1), RIPK3, SIRT2 (silent mating type information regulation 2 homolog), and MLKL (mixed lineage kinase domain-like protein) (Kaczmarek et al., 2013). Autophagy can be activated to block necroptosis in response to TNF-α, starvation or antigen stimulation for rapid T-cell clonal expansion (Bell et al., 2008). Once again, autophagy may facilitate necroptosis. In the presence of obatoclax (GX15-070), a small-molecule inhibitor of antiapoptotic Bcl-2 proteins, glucocorticoids-refractory acute lymphoblastic leukemia restored sensitivity to dexamethasone through rapid activation of autophagy-dependent cell death, with characteristic features of necroptosis (Bonapace et al., 2010). Some key signaling molecules may determine the final choice "autophagy or necroptosis." NAD-dependent deacetylase SIRT2 binds constitutively to RIP3 and is indispensable for the acetylation of RIP1. It is essential for the RIP1–RIP3 complex formation during necroapoptosis (Narayan et al., 2012). SIRT2 is a binding partner of FOXO1 while these two proteins dissociate under stress conditions. This leads to hyperacetylation of cytosolic FOXO1 which binds to Atg7 via its acetylated lysine residues and promote autophagy (Zhao et al., 2010).

Mitotic catastrophe (MC) is a type of cellular demise that occurs during mitosis due to severe cellular damage and deficient cell-cycle checkpoints (Castedo et al., 2004). Autophagy prevents cells undergoing MC from plunging into cell death (Lee et al., 2014). Alternatively, sublethal concentrations of DNA-damaging drugs could trigger ATG5 upregulation and translocation to the nucleus for MC induction, while autophagy blockade does not affect ATG5-dependent MC (Maskey et al., 2013).

Although senescence induction improves long-term therapeutic effect, potentially harmful properties of senescent tumor cells make their thorough elimination an urgent need (Dorr et al., 2013). Blockade of autophagy results in a senescence-to-apoptosis switch in KRAS-mutant NSCLC cells treated with irradiation and epidermal growth factor receptor (EGFR) inhibitors (Wang et al., 2014). Persistence of dormant drug-resistant cancer cells

following primary chemotherapy is a high risk of relapse and an obstacle for long-term survival. Although autophagy limits cancer progression by maintaining tumor cell dormancy, dormant state may help cancer cells to overcome the stress of ECM detachment, and subsequently resist to anoikis (White et al., 2004).

Cross talk between autophagy and different cell death modalities exists at many levels. Autophagy may be involved in, promote, suppress, or merely accompany lethal signaling. Thus, further studies are required to guide autophagy regulation during cancer therapy.

Decisive Factors for the Role of Autophagy in Cancer Therapy

The seemingly incompatible pro-survival versus cytotoxic functions of autophagy coexist in cancer therapy, raising the question about its net outcome. The extent of the damage and the threshold of tolerance in various cell types may decide whether autophagy manifests cytoprotection or cytotoxicity. The preferentially activated autophagy and cell death signaling network under differed circumstances should also be an important determinant.

The function of autophagy may also rely on the stage of autophagy process. Malignant glioma, characterized by aggressive cellular proliferation, diffuse infiltration, and resistance to cell death, usually associates with poor prognosis. Blocking autophagy at an early stage before conversion of LC3-I to LC3-II with a class III PI3K inhibitor 3-MA, or knock down of *Atg5*, *Atg10*, or *Atg12* suppress cytotoxic effect of imatinib against glioma. In contrast, vacuolar-type H^+-ATPase inhibitors BafA1 or RTA 203, which inhibit autophagy at a late stage, increased sensitivity of glioma to imatinib (Shingu et al., 2009).

Tumor microenvironment may play a decisive role in the function of autophagy. Hypoxic tumor microenvironment could trigger elevated autophagy and facilitate tumor resistance to ionizing radiation (He et al., 2012). A Ras-related tumor-suppressor gene ARHI (Aplasia Ras homolog member I), which is downregulated in more than 60% of ovarian cancers, could trigger autophagy and cell death when reintroduced into malignant cells. Unexpectedly, in the presence of tumor-promoting factors (e.g., growth factors, angiogenesis factors), ARHI-induced autophagy may also enable a state of dormancy and contribute to tumor recurrence and progression (Lu et al., 2008).

Additionally, the effect of autophagy-related immune response should not be ignored. Depending on the stress conditions and cell types in which autophagy takes place, autophagy may positively or negatively affect tumor immunity. We will discuss this aspect in the following part.

AUTOPHAGY AND TUMOR IMMUNITY

Tumor Cell Autophagy and Immunogenicity

Exposure or release of DAMP could render tumor cell death immunogenic, meaning capable of mounting an effective antitumor immunity. Secretion of "find me signal" ATP, alarmin HMGB1, and premortal surface exposure of CRT (calreticulin) have been reported as critical for recruiting antigen-presenting cells (APCs), facilitating their differentiation, maturation, phagocytosis, antigen processing, and presentation, which are

indispensable for the activation of cytotoxic T cells (Kroemer et al., 2013; Ma et al., 2013a). Autophagy is often enhanced after anticancer therapies and it may regulate DAMP release and degradation. Genetic ablation of *Atg5* and *Atg7*, or silencing their expression, led to autophagy deficiency and diminished ATP release from dying tumor cells upon chemotherapy both *in vitro* and *in vivo*. Autophagy-deficient tumors implanted in immunocompetent mice manifested hampered intratumoral dendritic cell (DC) accumulation, T-cell response failure, and resistant to chemotherapy (Michaud et al., 2011). Interestingly, overexpressing ATP degrading enzyme CD39 in tumor cells phenocopied autophagy-deficient tumors (Ma et al., 2013a), while intratumoral injection of ARL67156, an inhibitor of apyrases, can artificially increase extracellular ATP concentrations and restore chemosensitivity of autophagy-deficient tumors (Michaud et al., 2011). Autophagy inhibition was reported to facilitate radiosensitization and block clonogenic survival *in vitro*. Consistently, autophagy potentiates tumor-limiting effect of radiotherapy *in vivo*, but only in immunodeficient mice. When autophagy-deficient tumors were developed in the host harboring a competent immune system, however, they responded less well to radiotherapy due to reduced ATP release and hampered antitumor immunity (Ko et al., 2014). LAMP2A is an essential lysosomal protein which mediates chaperone-mediated autophagy (CMA). It was reported that the absence of LAMP2A completely abolished ecto-CRT emission, whereas *ATG5* knockdown increased the induction of ecto-CRT after treatment, suggesting that CMA and macroautophagy may differentially regulate CRT exposure (Garg et al., 2013a). In contrast, ROS-induced autophagy was shown to attenuate ecto-CRT induction and weaken immunogenicity of dying cancer cells upon hypericin-mediated photodynamic therapy (Garg et al., 2013b). Trehalose or EGFR-targeted diphtheria toxin administration was reported to activate autophagy in glioblastoma cells. Surprisingly, HMGB1-positive vesicles colocalized with $LC3^+$ cytoplasmic puncta after autophagy induction and the release of HMGB1 depended on autophagy-related gene expression and autophagosome formation (Thorburn et al., 2009).

Tumor cells undergo autophagy may display a different profile of tumor antigen. Vitamin E derivative alpha-tocopheryloxyacetic acid (α-TEA) treated mammary and lung cancer cells displayed typical characteristic features of autophagy and efficiently cross-primed antigen-specific $CD8^+$ T cells. Furthermore, α-TEA-generated autophagosomes act as a potent tumor vaccine (Li et al., 2012). Tumor-derived autophagosomes also induce B-cells activation, antibody production, and cytokine secretion, in addition to antigen cross-presentation to activate T cells (Li et al., 2013). Autophagy can impact tumor cell immunopeptidome through modulating translation, miRNA homeostasis, and the route of defective ribosomal initiation products (DRiPs) degradation (Ma et al., 2013a). Constitutively active autophagosomes and autophagic flux may contribute to create cancer-specific CTL epitopes (a process called "epitope liberation") (Demachi-Okamura et al., 2012).

Autophagy may also vary tumor sensitivity to immune destruction. Autophagy induction in tumor under hypoxia decreased tumor cell killing by cytotoxic T lymphocytes through STAT3 phosphorylation (Noman et al., 2011). Furthermore, hypoxic cancer cells could utilize autophagy process to degrade NK-derived lytic molecule granzyme B, while genetic inhibition of key autophagy proteins (e.g., BECN1, ATG5) is sufficient to restore their sensitivity to NK-mediated lysis (Baginska et al., 2013). As an important effector of antibody-dependent cellular cytotoxicity, FcαRI-expressing neutrophils could kill tumor

cells in an autophagy-dependent manner in the presence of tumor antigen-specific mAbs (Bakema et al., 2011).

Collectively, autophagy could determine the immunogenicity of cancer cells through regulating their secretome and surface proteome after cell stress, or their susceptibility to death induction.

Autophagy Regulates Immune Response

Autophagy taking place within immune cells was shown to affect their differentiation. High autophagic activity is a general phenomenon of stem cells under physiological conditions. Conditional deletion of *Atg7* in the hematopoietic system (Vav-*Atg7*$^{-/-}$ mice) leads to the accumulation of mitochondria and ROS, as well as increased DNA damage, apoptosis, and proliferation in the hematopoietic stem and progenitor cell (HSPC) compartment. Vav-*Atg7*$^{-/-}$ mice display crippled HSC activity and reduced production of several downstream cells, including common lymphoid progenitors, myeloid progenitor compartment, and immature NK cells (Mortensen et al., 2011).

Autophagy has both pro-death and pro-survival roles in immune cells. In T cells lacking caspase-8 or FADD activity, autophagy acts as an important cell death pathway (Bell et al., 2008). Autophagosomes are hardly detectable in naive CD4$^+$ T cells. However, autophagy can be observed in activated CD4$^+$ T cells upon TCR engagement, cytokine stimulation, and prolonged serum starvation. Blocking autophagy suppresses Th2 cell death upon growth factor withdrawal (Li et al., 2006). Also, CD4$^+$ T cells polarized to Th1, Th2, and Th17 lineages show remarkably different susceptibility to autophagy inhibition (Kovacs et al., 2012). In contrast, Atg5 knockout reduces the number of thymocytes and peripheral T cells. *Atg5*-deficient T cells undergo apoptosis to a greater extent compared with wild-type controls upon TCR triggering (Pua et al., 2007). Circulating monocytes are short lived (approximate 3-day or 1-day of half-life in humans or mice) and they are programmed to apoptosis without stimulation. In the presence of inflammatory factors, they activate pro-survival autophagy pathways, migrate into distinct tissues, and differentiate into macrophages or DCs (Zhang et al., 2012). When exposed to colony-stimulating factors, such as M-CSF or GM-CSF, autophagy is induced in monocytes via increased expression and phosphorylation of ULK1, or by activated MAPK/JNK and blocked Atg5 cleavage that release BECLIN1 from Bcl-2 (Zhang et al., 2012).

Autophagy could play a role in immune cell migration. Besides acting as a potent chemoattractants, CCL2 is able to induce hyperactivation of autophagy in monocytes and protect them against apoptosis in the tumor microenvironment, thus suggesting a role of autophagy in CCL2-induced monocyte recruitment (Roca et al., 2009). Immune cells rely on integrins to penetrate into tissues and to make contacts with other cells (Evans et al., 2009). It was reported that autophagy could modulate cell migration through degrading internalized β1 integrins and preventing its membrane recycling (Tuloup-Minguez et al., 2013).

Autophagy participates in the functional polarization of immune cells. Macrophages can be divided into immunostimulatory M1 phenotype and immunosuppressive M2 phenotype. NF-κB activity in tumor-associated macrophages (TAMs) plays a central role in macrophage polarization (Biswas and Lewis, 2010). Hepatoma-derived TLR2-related ligands are capable of supporting M2 macrophage differentiation through autophagy-mediated NF-κB

p65 proteolysis. Indeed, in the presence of hepatoma tumor cell condition medium, bone marrow–derived macrophages differentiate into a M2-like phenotype. And most isolated TAMs from hepatoma express a M2 phenotype with less NF-κB activation and are highly immunosuppressive (Chang et al., 2013). Knockdown of the mTOR-repressor TSC2 is critical for macrophage polarization toward the pro-tumoral M2 phenotype (Chen et al., 2012). Lysosomal cysteine proteases Cathepsin S, which is restrictedly expressed in lymphatic tissue, macrophages, and other APCs, serves as a bad prognostic marker in cancer patients. Recently, Cathepsin S was discovered to be indispensable for the fusion of autophagosomes with lysosomes and the completion of autophagy flux in TAM. Moreover, Cathepsin S deficiency blocks the M2 macrophage polarization and tumor progression (Yang et al., 2014).

Autophagy plays an important role in antigen processing and presentation. Tumor cells succumb to death after chemotherapy and release various DAMP, which could induce TIM-4 expression on tumor-associated macrophages and DCs. TIM-4 was found to directly interact with AMPKα1 and activate autophagy-mediated degradation of ingested tumors, thus impeding antigen presentation and CTL responses (Baghdadi et al., 2013). Autophagy contributes to the access of cytosolic, lysosomal, mitochondrial, membrane, or nuclear proteins to the MIIC (MHC class II loading compartment) for MHC class II restricted presentation (Dengjel et al., 2005). *Atg5* was found to be essential for DCs to present soluble and cell-associated antigens on MHC class II molecules for optimal $CD4^+$ T-cell responses (Lee et al., 2010). Also, $Atg5^{-/-}$ thymic medullary epithelial cells lose the ability to assist the positive and negative selection of $CD4^+$ T cells through MHC class II pathway (Nedjic et al., 2008). Amphisomes, which are formed upon the fusion of autophagosomes with endosomes, are critical for the antigen processing and presenting through MHC class I molecules (Fiegl et al., 2013). Both DCs and B cells can efficiently cross-prime tumor antigen–specific $CD8^+$ T cells, when loaded with tumor-derived autophagosomes DRibble (DRiPs-containing blebs), suggesting a potential therapeutic route of autophagy-dependent tumor vaccine (Li et al., 2011).

Autophagy also modulates soluble factor secretion. It may diminish IL-1β and IL-18 production through inflammasome limitation, either directly by degrading assembled inflammasomes (Shi et al., 2012) or indirectly by removing inflammasome activators ROS and mitochondrial DNA (Nakahira et al., 2011). Phosphatidylserine-displaying dead cells that engage TIM-4 for phagocytosis can be transiently decorated with LC3 for lysosome-mediated cargo degradation, a process called LC3-associated phagocytosis (LAP). LAP inhibits IL-1β and IL-6 production by macrophages, but enhances anti-inflammatory mediators such as IL-10 and TGF-β (Martinez et al., 2011). Autophagy could also participate in an unconventional secretory pathway and master the release of IL-1β (Dupont et al., 2011).

Immune Effectors Regulate Autophagy Induction

Multiple components of immune system could stimulate or inhibit autophagy. Extracellular or intracellular pattern recognition receptors, including TLRs, advanced glycation endproduct receptor, purinergic P2 receptors, and AIM2 (absent in melanoma 2) could perceive danger molecules, such as HMGB1, S100 proteins, ATP, and histone–DNA complexes, and activate autophagy (Tang et al., 2012). It was reported that several TLR ligands (e.g., TLR3, TLR4, TLR7 ligands) could induce autophagy. Single-stranded RNA engages

TLR7 and signals through MyD88 to induce autophagy for intracellular microbe elimination (Delgado et al., 2008). In DEN-induced liver injury model (diethylnitrosamine), TLR2 deficiency leads to reduced cell death, failure of senescence, suppression of autophagy flux in the liver, and increases susceptibility to HCC. Genetic deletion of *TLR2* blocks ASK1/p38 MAPK/NF-κB signaling and cytokine production (e.g., IFN-γ, IL-1α, IL-1β, TNF-α). Interestingly, prophylactic treatment with a Th1 cytokine IFN-γ restored senescence and autophagy flux, as well as attenuated HCC incidence (Lin et al., 2013). IFN-γ enhances autophagy via increased expression of BECLIN1 and inhibits *Helicobacter* infection as well as carcinogenesis in the gastric mucosa (Tu et al., 2011). In sharp contrast, Th2 cytokines, including IL-4 and IL-13, as well as the anti-inflammatory mediator IL-10, inhibit autophagy (Ma et al., 2013b). Notably, at high effector/target ratios, lymphocytes could induce cell-mediated autophagy (C-MA) in the remaining target cells and promote cancer cell survival. Cell-to-cell contact strongly enhances C-MA while cytokines also adjust this process. NK cells, macrophages, and T cells have been proved as executors of C-MA (Buchser et al., 2012).

THERAPEUTIC STRATEGIES TARGETING AUTOPHAGY

Autophagy pathway can be targeted at multiple levels for its regulation. As autophagy plays both protective and destructive roles in oncogenesis and cancer treatment, it is essential to develop therapeutic agents which can positively and negatively modulate this process. High-throughput screening of chemical libraries is ongoing in order to identify small molecules that can potently and specifically activate or inhibit autophagy.

Tools for Autophagy Induction

Inhibition of mTOR or mTORC1 is a major strategy to induce autophagy. A lipophilic macrolide antibiotic rapamycin (also known as sirolimus) forms a complex with the immunophilin FKBP12 (FK506-binding protein of 12 kDa) and stabilizes the stoichiometric association of mTOR with raptor (Kim et al., 2002). Rapamycin inhibits both mTORC1 and its downstream effectors S6K1 (ribosomal protein S6 kinase 1) and eIF4E (eukaryotic translation initiation factor 4E). Rapamycin and its analogs temsirolimus (CCI-779), everolimus (RAD-001), and deforolimus (AP-23573) are strong inducers of autophagy (Rubinsztein et al., 2012). Clinical approved drugs (perhexilene, niclosamide, amiodarone) and a pharmacological reagent (rottlerin) were also identified as mTORC1 inhibitor and autophagy stimulator in an automated cell-based assay (Balgi et al., 2009). Compared to the rapalogs, a new class of ATP-competitive mTOR inhibitor Torin2 shows potent dual blockade of mTORC1 and mTORC2 and exerts broad antitumor activity (Liu et al., 2013).

The lipid kinases phosphatidylinositol 3-kinases (PI3Ks) are important targets in autophagy regulation. Of the three major classes of PI3Ks, class I are expressed as heterodimers containing one p110 catalytic subunit and one regulatory subunit (Shanware et al., 2013). They mainly act as an autophagy suppressor, though p110β catalytic subunit has been reported as pro-autophagic (Dou et al., 2013). It can be activated by tyrosine kinase–based and heterotrimeric G protein–based signaling pathways and it preferentially converts the

plasma membrane lipid PIP2 (phosphatidylinositol 4,5-bisphosphate) to generate second messenger PIP3 (phosphatidylinositol 4,5-bisphosphate). The accumulation of PIP3 at the inner leaflet of cell membrane further recruits serine–threonine kinase AKT (also known as PKB) to the plasma membrane for autophagy inhibition, partially through mTOR. On the contrary, Class III PI3K complex accelerates the autophagy process. They are composed of one catalytic subunit VPS34 and one regulatory subunit VPS15. Class III enzyme mainly catalyzes the conversion of PI to PI3P (phosphatidylinositol 3-phosphate), which is essential for nucleation/autophagosome formation. The role of class II PI3Ks in autophagy is less well understood. They form monomers of catalytic subunits without known obligatory regulatory subunit. Class II enzyme utilizes PI and PIP2 as substrates to generate PIP3 and therefore contribute to autophagy induction (Kroemer et al., 2013).

Class I PI3K inhibition provides another promising therapeutic approach for autophagy induction. Pan-class I PI3K targeted drugs admitted to clinical trials against advanced solid tumors include: Buparlisib (BKM120), BAY80-6946, PX-866. Isoform-specific class I PI3K inhibitors targeting the four p110 subunits offer greater opportunities to customize therapy and are also under clinical investigation. MLN01117 (INK1117) is specific for p110α while GSK2636771 and SAR260301 show highly preferential inhibition to p110β (Dienstmann et al., 2014). Dual PI3K/mTOR inhibitors, such as BEZ-235, BGT-226, PF-04691502, GDC-0980, GSK-2126458, PF-05212384, XL-765, are also under clinical study in solid tumors, such as breast cancer, NSCLC, glioblastoma, and prostate cancer (Akinleye et al., 2013), and appear to have the broadest activity profile. AKT intervention drugs, including catalytic inhibitors (e.g., GDC-0068 and AZD5363) and allosteric inhibitors (e.g., MK-2206), may provide therapeutic advantages over PI3K inhibitors for tumors with either PTEN loss or PIK3CA mutations (Dienstmann et al., 2014).

Tools for Autophagy Inhibition

Early stages of autophagy can be blocked through inhibiting the class III PI3K and disabling the formation of autophagosome. Pharmaceutical inhibitors for class III PI3K include: 3-MA, wortmannin, and LY294002, which are mildly poisonous in animal studies and are not advanced to clinical evaluation. Some orally bioavailable agents, including idelalisib (CAL-101), buparlisib (BKM 120), IPI-145, PX-866, GDC-0941, GDC-0032, and GDC-0941, are under various stages of clinical development and show favorable safety. Idelalisib is the most advanced candidate with impressive efficacy either used as a single agent or given in combination with standard therapies across multiple subtypes of non-Hodgkin's lymphoma.

Late stage of autophagy can be blocked through limiting the maturation and degradation of autophagic vacuoles. Microtubules participate in the autophagosome formation, trafficking, and fusion of autophagosomes with lysosomes or endosomes (Mackeh et al., 2013). Microtubule-disrupting agents, including nocodazole, colchicine, and vinblastine, and intracellular protein transport blocker (e.g., monensin) can prevent the fusion of the autophagosome with the lysosome (Shen et al., 2011). Macrolide antibiotic BafA1 selectively inhibits vacuolar H^+ ATPase to prevent lysosome acidification and autophagosome–lysosome fusion. Lysosomotropic agents CQ (chloroquine) and HCQ (hydroxychloroquine)

could increase lysosomal pH and prevent the digestive activity of hydrolases (Amaravadi et al., 2011). Inhibitors of lysosomal proteolysis, such as leupeptin and pepstatin, could also block the digestion of autophagic cargo. A small-molecule inhibitor of heat shock protein 70, 2-phenylethynesulfonamide, could impair autophagy, trigger protein aggregation, and induce apoptotic tumor cell death through blocking lysosomal functions (Leu et al., 2009). Another specific and potent autophagy inhibitor spautin-1 is able to inhibit two ubiquitin-specific peptidases, USP10 and USP13, which regulate the deubiquitination of BECLIN1 in VPS34 complexes (Liu et al., 2011).

DISCUSSION

Although basic, preclinical, and clinical studies suggest that autophagy can be targeted as an Achilles' heel in cancer, several critical points should be addressed in order to achieve optimal therapeutic effect. The dual role of autophagy both in tumorigenesis and upon cancer treatment highlights the need of careful decipher its role in varied settings. Before applying autophagy-targeted treatments, clinical oncologists and cancer researchers have to understand whether autophagy level changes in tumor sites, rather than healthy tissues, are the sign of tumor cell survival or cell death, or indicate responsiveness or resistance to therapy. The method to quickly monitor autophagy level within patient tumor microenvironment remains a challenge. Analyzing the role of autophagy in each cancer type (especially under current therapy regimens) could provide the basis for therapeutic optimization. Mapping the interactome of core autophagy molecules (e.g., metabolism, cell death signaling pathways, and DAMP-generating machinery) may provide reliable parameters that are capable of predicting beneficial effect of autophagy interventions. However, non-autophagic roles of autophagy proteins should not be ignored during therapy designing. Targeting components of autophagy pathway does not necessary function through regulating autophagy process.

As only a certain fraction of tumor cells undergoes autophagy, autophagy modulators are more likely to be used in combination with other cancer therapies, rather than as single agents. Among more than 50 clinical trials registered in the NCI to evaluate effect of HCQ, CQ, or quinacrine as an autophagy inhibitor in a variety of human cancers (http://clinicaltrials.gov), the great majority apply the combination strategy. Therefore, the timing of adding autophagy modulators in other therapy regimens, possible drug–drug interactions, pharmacokinetic, and pharmacodynamics should be taken into serious consideration.

Autophagy-mediated modulation of immune response is of fundamental importance. It is now clearly established that autophagy plays a crucial role in increasing the immunogenicity of tumor cells and actively participates in immune cell differentiation, migration, functional polarization, antigen processing, effector T-cell activation, and cytokine secretion. Therefore, immune suppression may be a deleterious side effect of autophagy inhibition, while autophagy induction could be an important strategy for cancer prevention. Efforts could be made to develop pharmaceutical tools which specifically target tumor or immune cells with minimal interference on normal tissues.

References

Acosta, J.C., O'Loghlen, A., Banito, A., et al., 2008. Chemokine signaling via the CXCR2 receptor reinforces senescence. Cell 133, 1006–1018.

Akinleye, A., Avvaru, P., Furqan, M., et al., 2013. Phosphatidylinositol 3-kinase (PI3K) inhibitors as cancer therapeutics. J. Hematol. Oncol. 6, 88.

Amaravadi, R.K., Yu, D., Lum, J.J., et al., 2007. Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. J. Clin. Invest. 117, 326–336.

Amaravadi, R.K., Lippincott-Schwartz, J., Yin, X.M., et al., 2011. Principles and current strategies for targeting autophagy for cancer treatment. Clin. Cancer Res. 17, 654–666.

Apel, A., Herr, I., Schwarz, H., et al., 2008. Blocked autophagy sensitizes resistant carcinoma cells to radiation therapy. Cancer Res. 68, 1485–1494.

Baghdadi, M., Yoneda, A., Yamashina, T., et al., 2013. TIM-4 glycoprotein-mediated degradation of dying tumor cells by autophagy leads to reduced antigen presentation and increased immune tolerance. Immunity 39, 1070–1081.

Baginska, J., Viry, E., Berchem, G., et al., 2013. Granzyme B degradation by autophagy decreases tumor cell susceptibility to natural killer-mediated lysis under hypoxia. Proc. Natl. Acad. Sci. U.S.A. 110, 17450–17455.

Bakema, J.E., Ganzevles, S.H., Fluitsma, D.M., et al., 2011. Targeting FcalphaRI on polymorphonuclear cells induces tumor cell killing through autophagy. J. Immunol. 187, 726–732.

Balgi, A.D., Fonseca, B.D., Donohue, E., et al., 2009. Screen for chemical modulators of autophagy reveals novel therapeutic inhibitors of mTORC1 signaling. PLoS One 4, e7124.

Bartkova, J., Rezaei, N., Liontos, M., et al., 2006. Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. Nature 444, 633–637.

Bell, B.D., Leverrier, S., Weist, B.M., et al., 2008. FADD and caspase-8 control the outcome of autophagic signaling in proliferating T cells. Proc. Natl. Acad. Sci. U.S.A. 105, 16677–16682.

Biswas, S.K., Lewis, C.E., 2010. NF-kappaB as a central regulator of macrophage function in tumors. J. Leukoc. Biol. 88, 877–884.

Bonapace, L., Bornhauser, B.C., Schmitz, M., et al., 2010. Induction of autophagy-dependent necroptosis is required for childhood acute lymphoblastic leukemia cells to overcome glucocorticoid resistance. J. Clin. Invest. 120, 1310–1323.

Boya, P., Gonzalez-Polo, R.A., Casares, N., et al., 2005. Inhibition of macroautophagy triggers apoptosis. Mol. Cell. Biol. 25, 1025–1040.

Bristol, M.L., Di, X., Beckman, M.J., et al., 2012. Dual functions of autophagy in the response of breast tumor cells to radiation: cytoprotective autophagy with radiation alone and cytotoxic autophagy in radiosensitization by vitamin D3. Autophagy 8, 739–753.

Buchser, W.J., Laskow, T.C., Pavlik, P.J., et al., 2012. Cell-mediated autophagy promotes cancer cell survival. Cancer Res. 72, 2970–2979.

Byun, J.Y., Yoon, C.H., An, S., et al., 2009. The Rac1/MKK7/JNK pathway signals upregulation of Atg5 and subsequent autophagic cell death in response to oncogenic Ras. Carcinogenesis 30, 1880–1888.

Capparelli, C., Guido, C., Whitaker-Menezes, D., et al., 2012. Autophagy and senescence in cancer-associated fibroblasts metabolically supports tumor growth and metastasis via glycolysis and ketone production. Cell Cycle 11, 2285–2302.

Castedo, M., Perfettini, J.L., Roumier, T., et al., 2004. Cell death by mitotic catastrophe: a molecular definition. Oncogene 23, 2825–2837.

Chaachouay, H., Ohneseit, P., Toulany, M., et al., 2011. Autophagy contributes to resistance of tumor cells to ionizing radiation. Radiother. Oncol. 99, 287–292.

Chang, C.P., Su, Y.C., Hu, C.W., et al., 2013. TLR2-dependent selective autophagy regulates NF-kappaB lysosomal degradation in hepatoma-derived M2 macrophage differentiation. Cell Death Differ. 20, 515–523.

Chen, W., Ma, T., Shen, X.N., et al., 2012. Macrophage-induced tumor angiogenesis is regulated by the TSC2-mTOR pathway. Cancer Res. 72, 1363–1372.

Choi, A.M., Ryter, S.W., Levine, B., 2013. Autophagy in human health and disease. N. Engl. J. Med. 368, 651–662.

Debnath, J., 2008. Detachment-induced autophagy during anoikis and lumen formation in epithelial acini. Autophagy 4, 351–353.

Degenhardt, K., Mathew, R., Beaudoin, B., et al., 2006. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. Cancer Cell 10, 51–64.
Delgado, M.A., Elmaoued, R.A., Davis, A.S., et al., 2008. Toll-like receptors control autophagy. EMBO J. 27, 1110–1121.
Demachi-Okamura, A., Torikai, H., Akatsuka, Y., et al., 2012. Autophagy creates a CTL epitope that mimics tumor-associated antigens. PLoS One 7, e47126.
Dengjel, J., Schoor, O., Fischer, R., et al., 2005. Autophagy promotes MHC class II presentation of peptides from intracellular source proteins. Proc. Natl. Acad. Sci. U.S.A. 102, 7922–7927.
Dienstmann, R., Rodon, J., Serra, V., et al., 2014. Picking the point of inhibition: a comparative review of PI3K/AKT/mTOR pathway inhibitors. Mol. Cancer Ther. 13, 1021–1031.
Dorr, J.R., Yu, Y., Milanovic, M., et al., 2013. Synthetic lethal metabolic targeting of cellular senescence in cancer therapy. Nature 501, 421–425.
Dou, Z., Pan, J.A., Dbouk, H.A., et al., 2013. Class IA PI3K p110beta subunit promotes autophagy through Rab5 small GTPase in response to growth factor limitation. Mol. Cell 50, 29–42.
Dupont, N., Jiang, S., Pilli, M., et al., 2011. Autophagy-based unconventional secretory pathway for extracellular delivery of IL-1beta. EMBO J. 30, 4701–4711.
Evans, R., Patzak, I., Svensson, L., et al., 2009. Integrins in immunity. J. Cell Sci. 122, 215–225.
Ewald, J.A., Desotelle, J.A., Wilding, G., et al., 2010. Therapy-induced senescence in cancer. J. Natl. Cancer Inst. 102, 1536–1546.
Fang, H., Liu, A., Dahmen, U., et al., 2013. Dual role of chloroquine in liver ischemia reperfusion injury: reduction of liver damage in early phase, but aggravation in late phase. Cell Death Dis. 4, e694.
Feng, Z., Hu, W., de Stanchina, E., et al., 2007. The regulation of AMPK beta1, TSC2, and PTEN expression by p53: stress, cell and tissue specificity, and the role of these gene products in modulating the IGF-1-AKT-mTOR pathways. Cancer Res. 67, 3043–3053.
Fiegl, D., Kagebein, D., Liebler-Tenorio, E.M., et al., 2013. Amphisomal route of MHC class I cross-presentation in bacteria-infected dendritic cells. J. Immunol. 190, 2791–2806.
Fujii, S., Mitsunaga, S., Yamazaki, M., et al., 2008. Autophagy is activated in pancreatic cancer cells and correlates with poor patient outcome. Cancer Sci. 99, 1813–1819.
Garg, A.D., Dudek, A.M., Agostinis, P., 2013a. Calreticulin surface exposure is abrogated in cells lacking, chaperone-mediated autophagy-essential gene, LAMP2A. Cell Death Dis. 4, e826.
Garg, A.D., Dudek, A.M., Ferreira, G.B., et al., 2013b. ROS-induced autophagy in cancer cells assists in evasion from determinants of immunogenic cell death. Autophagy 9, 1292–1307.
Gewirtz, D.A., 2009. Autophagy, senescence and tumor dormancy in cancer therapy. Autophagy 5, 1232–1234.
Giatromanolaki, A., Koukourakis, M.I., Sowter, H.M., et al., 2004. BNIP3 expression is linked with hypoxia-regulated protein expression and with poor prognosis in non-small cell lung cancer. Clin. Cancer Res. 10, 5566–5571.
Giatromanolaki, A., Koukourakis, M.I., Gatter, K.C., et al., 2008. BNIP3 expression in endometrial cancer relates to active hypoxia inducible factor 1alpha pathway and prognosis. J. Clin. Pathol. 61, 217–220.
Goehe, R.W., Di, X., Sharma, K., et al., 2012. The autophagy-senescence connection in chemotherapy: must tumor cells (self) eat before they sleep? J. Pharmacol. Exp. Ther. 343, 763–778.
Grivennikov, S.I., Greten, F.R., Karin, M., 2010. Immunity, inflammation, and cancer. Cell 140, 883–899.
Guo, J.Y., Chen, H.Y., Mathew, R., et al., 2011. Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis. Genes Dev. 25, 460–470.
Han, C., Sun, B., Wang, W., et al., 2011. Overexpression of microtubule-associated protein-1 light chain 3 is associated with melanoma metastasis and vasculogenic mimicry. Tohoku J. Exp. Med. 223, 243–251.
Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. Cell 144, 646–674.
He, W.S., Dai, X.F., Jin, M., et al., 2012. Hypoxia-induced autophagy confers resistance of breast cancer cells to ionizing radiation. Oncol. Res. 20, 251–258.
Hu, Y.L., DeLay, M., Jahangiri, A., et al., 2012. Hypoxia-induced autophagy promotes tumor cell survival and adaptation to antiangiogenic treatment in glioblastoma. Cancer Res. 72, 1773–1783.
Johansen, T., Lamark, T., 2011. Selective autophagy mediated by autophagic adapter proteins. Autophagy 7, 279–296.
Kaczmarek, A., Vandenabeele, P., Krysko, D.V., 2013. Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. Immunity 38, 209–223.

Kang, R., Zeh, H.J., Lotze, M.T., et al., 2011. The Beclin 1 network regulates autophagy and apoptosis. Cell Death Differ. 18, 571–580.
Kanzawa, T., Zhang, L., Xiao, L., et al., 2005. Arsenic trioxide induces autophagic cell death in malignant glioma cells by upregulation of mitochondrial cell death protein BNIP3. Oncogene 24, 980–991.
Karnoub, A.E., Weinberg, R.A., 2008. Ras oncogenes: split personalities. Nat. Rev. Mol. Cell Biol. 9, 517–531.
Kim, D.H., Sarbassov, D.D., Ali, S.M., et al., 2002. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell 110, 163–175.
Kim, J., Kundu, M., Viollet, B., et al., 2011a. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat. Cell Biol. 13, 132–141.
Kim, J.H., Kim, H.Y., Lee, Y.K., et al., 2011b. Involvement of mitophagy in oncogenic K-Ras-induced transformation: overcoming a cellular energy deficit from glucose deficiency. Autophagy 7, 1187–1198.
Kisen, G.O., Tessitore, L., Costelli, P., et al., 1993. Reduced autophagic activity in primary rat hepatocellular carcinoma and ascites hepatoma cells. Carcinogenesis 14, 2501–2505.
Ko, A., Kanehisa, A., Martins, I., et al., 2014. Autophagy inhibition radiosensitizes *in vitro*, yet reduces radioresponses *in vivo* due to deficient immunogenic signalling. Cell Death Differ. 21, 92–99.
Kondo, Y., Kondo, S., 2006. Autophagy and cancer therapy. Autophagy 2, 85–90.
Kovacs, J.R., Li, C., Yang, Q., et al., 2012. Autophagy promotes T-cell survival through degradation of proteins of the cell death machinery. Cell Death Differ. 19, 144–152.
Kroemer, G., Jaattela, M., 2005. Lysosomes and autophagy in cell death control. Nat. Rev. Cancer 5, 886–897.
Kroemer, G., Galluzzi, L., Kepp, O., et al., 2013. Immunogenic cell death in cancer therapy. Annu. Rev. Immunol. 31, 51–72.
Kuwahara, Y., Oikawa, T., Ochiai, Y., et al., 2011. Enhancement of autophagy is a potential modality for tumors refractory to radiotherapy. Cell Death Dis. 2, e177.
Lee, H.K., Mattei, L.M., Steinberg, B.E., et al., 2010. *In vivo* requirement for Atg5 in antigen presentation by dendritic cells. Immunity 32, 227–239.
Lee, S.Y., Oh, J.S., Rho, J.H., et al., 2014. Retinal pigment epithelial cells undergoing mitotic catastrophe are vulnerable to autophagy inhibition. Cell Death Dis. 5, e1303.
Leu, J.I., Pimkina, J., Frank, A., et al., 2009. A small molecule inhibitor of inducible heat shock protein 70. Mol. Cell 36, 15–27.
Levy, J.M., Thorburn, A., 2011. Targeting autophagy during cancer therapy to improve clinical outcomes. Pharmacol. Ther. 131, 130–141.
Li, C., Capan, E., Zhao, Y., et al., 2006. Autophagy is induced in CD4+ T cells and important for the growth factor-withdrawal cell death. J. Immunol. 177, 5163–5168.
Li, W., Zhou, M., Ren, H., et al., 2013. Tumor-derived autophagosomes (DRibbles) induce B cell activation in a TLR2-MyD88 dependent manner. PLoS One 8, e53564.
Li, Y., Wang, L.X., Pang, P., et al., 2011. Tumor-derived autophagosome vaccine: mechanism of cross-presentation and therapeutic efficacy. Clin. Cancer Res. 17, 7047–7057.
Li, Y., Hahn, T., Garrison, K., et al., 2012. The vitamin E analogue alpha-TEA stimulates tumor autophagy and enhances antigen cross-presentation. Cancer Res. 72, 3535–3545.
Lin, H., Yan, J., Wang, Z., et al., 2013. Loss of immunity-supported senescence enhances susceptibility to hepatocellular carcinogenesis and progression in Toll-like receptor 2-deficient mice. Hepatology 57, 171–182.
Liu, J., Xia, H., Kim, M., et al., 2011. Beclin1 controls the levels of p53 by regulating the deubiquitination activity of USP10 and USP13. Cell 147, 223–234.
Liu, Q., Xu, C., Kirubakaran, S., et al., 2013. Characterization of Torin2, an ATP-competitive inhibitor of mTOR, ATM, and ATR. Cancer Res. 73, 2574–2586.
Lomonaco, S.L., Finniss, S., Xiang, C., et al., 2009. The induction of autophagy by gamma-radiation contributes to the radioresistance of glioma stem cells. Int. J. Cancer 125, 717–722.
Lu, Z., Luo, R.Z., Lu, Y., et al., 2008. The tumor suppressor gene ARHI regulates autophagy and tumor dormancy in human ovarian cancer cells. J. Clin. Invest. 118, 3917–3929.
Ma, Y., Adjemian, S., Mattarollo, S.R., et al., 2013a. Anticancer chemotherapy-induced intratumoral recruitment and differentiation of antigen-presenting cells. Immunity 38, 729–741.
Ma, Y., Galluzzi, L., Zitvogel, L., et al., 2013b. Autophagy and cellular immune responses. Immunity 39, 211–227.
Mackeh, R., Perdiz, D., Lorin, S., et al., 2013. Autophagy and microtubules—new story, old players. J. Cell Sci. 126, 1071–1080.

Marino, G., Salvador-Montoliu, N., Fueyo, A., et al., 2007. Tissue-specific autophagy alterations and increased tumorigenesis in mice deficient in Atg4C/autophagin-3. J. Biol. Chem. 282, 18573–18583.
Martinez, J., Almendinger, J., Oberst, A., et al., 2011. Microtubule-associated protein 1 light chain 3 alpha (LC3)-associated phagocytosis is required for the efficient clearance of dead cells. Proc. Natl. Acad. Sci. U. S. A. 108, 17396–17401.
Maskey, D., Yousefi, S., Schmid, I., et al., 2013. ATG5 is induced by DNA-damaging agents and promotes mitotic catastrophe independent of autophagy. Nat. Commun. 4, 2130.
Mathew, R., Kongara, S., Beaudoin, B., et al., 2007. Autophagy suppresses tumor progression by limiting chromosomal instability. Genes Dev. 21, 1367–1381.
Mathew, R., Karp, C.M., Beaudoin, B., et al., 2009. Autophagy suppresses tumorigenesis through elimination of p62. Cell 137, 1062–1075.
Michaud, M., Martins, I., Sukkurwala, A.Q., et al., 2011. Autophagy-dependent anticancer immune responses induced by chemotherapeutic agents in mice. Science 334, 1573–1577.
Molejon, M.I., Ropolo, A., Vaccaro, M.I., 2013. VMP1 is a new player in the regulation of the autophagy-specific phosphatidylinositol 3-kinase complex activation. Autophagy 9, 933–935.
Mortensen, M., Soilleux, E.J., Djordjevic, G., et al., 2011. The autophagy protein Atg7 is essential for hematopoietic stem cell maintenance. J. Exp. Med. 208, 455–467.
Murrow, L., Debnath, J., 2013. Autophagy as a stress-response and quality-control mechanism: implications for cell injury and human disease. Annu. Rev. Pathol. 8, 105–137.
Nakahira, K., Haspel, J.A., Rathinam, V.A., et al., 2011. Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. Nat. Immunol. 12, 222–230.
Narayan, N., Lee, I.H., Borenstein, R., et al., 2012. The NAD-dependent deacetylase SIRT2 is required for programmed necrosis. Nature 492, 199–204.
Nedjic, J., Aichinger, M., Emmerich, J., et al., 2008. Autophagy in thymic epithelium shapes the T-cell repertoire and is essential for tolerance. Nature 455, 396–400.
Nikoletopoulou, V., Markaki, M., Palikaras, K., et al., 2013. Crosstalk between apoptosis, necrosis and autophagy. Biochim. Biophys. Acta 1833, 3448–3459.
Noman, M.Z., Janji, B., Kaminska, B., et al., 2011. Blocking hypoxia-induced autophagy in tumors restores cytotoxic T-cell activity and promotes regression. Cancer Res. 71, 5976–5986.
Ohsumi, Y., 2001. Molecular dissection of autophagy: two ubiquitin-like systems. Nat. Rev. Mol. Cell Biol. 2, 211–216.
Pua, H.H., Dzhagalov, I., Chuck, M., et al., 2007. A critical role for the autophagy gene Atg5 in T cell survival and proliferation. J. Exp. Med. 204, 25–31.
Qu, X., Yu, J., Bhagat, G., et al., 2003. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. J. Clin. Invest. 112, 1809–1820.
Rao, S., Tortola, L., Perlot, T., et al., 2014. A dual role for autophagy in a murine model of lung cancer. Nat. Commun. 5, 3056.
Reggiori, F., Klionsky, D.J., 2006. Atg9 sorting from mitochondria is impaired in early secretion and VFT-complex mutants in *Saccharomyces cerevisiae*. J. Cell Sci. 119, 2903–2911.
Roca, H., Varsos, Z.S., Sud, S., et al., 2009. CCL2 and interleukin-6 promote survival of human CD11b+ peripheral blood mononuclear cells and induce M2-type macrophage polarization. J. Biol. Chem. 284, 34342–34354.
Rolland, P., Madjd, Z., Durrant, L., et al., 2007. The ubiquitin-binding protein p62 is expressed in breast cancers showing features of aggressive disease. Endocr. Relat. Cancer 14, 73–80.
Rosenfeldt, M.T., O'Prey, J., Morton, J.P., et al., 2013. p53 status determines the role of autophagy in pancreatic tumour development. Nature 504, 296–300.
Rouschop, K.M., van den Beucken, T., Dubois, L., et al., 2010. The unfolded protein response protects human tumor cells during hypoxia through regulation of the autophagy genes MAP1LC3B and ATG5. J. Clin. Invest. 120, 127–141.
Rubinsztein, D.C., Codogno, P., Levine, B., 2012. Autophagy modulation as a potential therapeutic target for diverse diseases. Nat. Rev. Drug Discov. 11, 709–730.
Scherz-Shouval, R., Weidberg, H., Gonen, C., et al., 2010. p53-dependent regulation of autophagy protein LC3 supports cancer cell survival under prolonged starvation. Proc. Natl. Acad. Sci. U.S.A. 107, 18511–18516.
Schmukler, E., Kloog, Y., Pinkas-Kramarski, R., 2014. Ras and autophagy in cancer development and therapy. Oncotarget 5, 577–586.

Schreiber, R.D., Old, L.J., Smyth, M.J., 2011. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science 331, 1565–1570.
Schwarze, P.E., Seglen, P.O., 1985. Reduced autophagic activity, improved protein balance and enhanced *in vitro* survival of hepatocytes isolated from carcinogen-treated rats. Exp. Cell Res. 157, 15–28.
Seglen, P.O., Schwarze, P.E., Saeter, G., 1986. Changes in cellular ploidy and autophagic responsiveness during rat liver carcinogenesis. Toxicol. Pathol. 14, 342–348.
Shanware, N.P., Bray, K., Abraham, R.T., 2013. The PI3K, metabolic, and autophagy networks: interactive partners in cellular health and disease. Annu. Rev. Pharmacol. Toxicol. 53, 89–106.
Shen, S., Kepp, O., Michaud, M., et al., 2011. Association and dissociation of autophagy, apoptosis and necrosis by systematic chemical study. Oncogene 30, 4544–4556.
Shi, C.S., Shenderov, K., Huang, N.N., et al., 2012. Activation of autophagy by inflammatory signals limits IL-1beta production by targeting ubiquitinated inflammasomes for destruction. Nat. Immunol. 13, 255–263.
Shingu, T., Fujiwara, K., Bogler, O., et al., 2009. Inhibition of autophagy at a late stage enhances imatinib-induced cytotoxicity in human malignant glioma cells. Int. J. Cancer 124, 1060–1071.
Singh, K., Matsuyama, S., Drazba, J.A., et al., 2012. Autophagy-dependent senescence in response to DNA damage and chronic apoptotic stress. Autophagy 8, 236–251.
Soussi, T., Wiman, K.G., 2007. Shaping genetic alterations in human cancer: the p53 mutation paradigm. Cancer Cell 12, 303–312.
Stumptner, C., Heid, H., Fuchsbichler, A., et al., 1999. Analysis of intracytoplasmic hyaline bodies in a hepatocellular carcinoma. Demonstration of p62 as major constituent. Am. J. Pathol. 154, 1701–1710.
Takahashi, Y., Coppola, D., Matsushita, N., et al., 2007. Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. Nat. Cell Biol. 9, 1142–1151.
Takamura, A., Komatsu, M., Hara, T., et al., 2011. Autophagy-deficient mice develop multiple liver tumors. Genes Dev. 25, 795–800.
Tang, D., Kang, R., Livesey, K.M., et al., 2010. Endogenous HMGB1 regulates autophagy. J. Cell Biol. 190, 881–892.
Tang, D., Kang, R., Coyne, C.B., et al., 2012. PAMPs and DAMPs: signal 0s that spur autophagy and immunity. Immunol. Rev. 249, 158–175.
Tasdemir, E., Maiuri, M.C., Galluzzi, L., et al., 2008. Regulation of autophagy by cytoplasmic p53. Nat. Cell Biol. 10, 676–687.
Thorburn, J., Horita, H., Redzic, J., et al., 2009. Autophagy regulates selective HMGB1 release in tumor cells that are destined to die. Cell Death Differ. 16, 175–183.
Tu, S.P., Quante, M., Bhagat, G., et al., 2011. IFN-gamma inhibits gastric carcinogenesis by inducing epithelial cell autophagy and T-cell apoptosis. Cancer Res. 71, 4247–4259.
Tuloup-Minguez, V., Hamai, A., Greffard, A., et al., 2013. Autophagy modulates cell migration and beta1 integrin membrane recycling. Cell Cycle 12, 3317–3328.
Tumbarello, D.A., Waxse, B.J., Arden, S.D., et al., 2012. Autophagy receptors link myosin VI to autophagosomes to mediate Tom1-dependent autophagosome maturation and fusion with the lysosome. Nat. Cell Biol. 14, 1024–1035.
Turcotte, S., Chan, D.A., Sutphin, P.D., et al., 2008. A molecule targeting VHL-deficient renal cell carcinoma that induces autophagy. Cancer Cell 14, 90–102.
Vandenabeele, P., Vanden Berghe, T., Festjens, N., 2006. Caspase inhibitors promote alternative cell death pathways. Sci. STKE 2006, pe44.
Vessoni, A.T., Muotri, A.R., Okamoto, O.K., 2012. Autophagy in stem cell maintenance and differentiation. Stem Cells Dev. 21, 513–520.
Voss, V., Senft, C., Lang, V., et al., 2010. The pan-Bcl-2 inhibitor (-)-gossypol triggers autophagic cell death in malignant glioma. Mol. Cancer Res. 8, 1002–1016.
Wang, M., Kern, A.M., Hulskotter, M., et al., 2014. EGFR-mediated chromatin condensation protects KRAS-mutant cancer cells against ionizing radiation. Cancer Res. 74, 2825–2834.
Wang, Y., Wang, X.D., Lapi, E., et al., 2012. Autophagic activity dictates the cellular response to oncogenic RAS. Proc. Natl. Acad. Sci. U.S.A. 109, 13325–13330.
White, D.E., Kurpios, N.A., Zuo, D., et al., 2004. Targeted disruption of beta1-integrin in a transgenic mouse model of human breast cancer reveals an essential role in mammary tumor induction. Cancer Cell 6, 159–170.
Wojtkowiak, J.W., Rothberg, J.M., Kumar, V., et al., 2012. Chronic autophagy is a cellular adaptation to tumor acidic pH microenvironments. Cancer Res. 72, 3938–3947.

Wu, S.Y., Lan, S.H., Cheng, D.E., et al., 2011. Ras-related tumorigenesis is suppressed by BNIP3-mediated autophagy through inhibition of cell proliferation. Neoplasia 13, 1171–1182.
Xue, W., Zender, L., Miething, C., et al., 2007. Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. Nature 445, 656–660.
Yang, M., Liu, J., Shao, J., et al., 2014. Cathepsin S-mediated autophagic flux in tumor-associated macrophages accelerate tumor development by promoting M2 polarization. Mol. Cancer 13, 43.
Young, A.R., Narita, M., Ferreira, M., et al., 2009. Autophagy mediates the mitotic senescence transition. Genes Dev. 23, 798–803.
Yousefi, S., Perozzo, R., Schmid, I., et al., 2006. Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. Nat. Cell Biol. 8, 1124–1132.
Zhang, Y., Morgan, M.J., Chen, K., et al., 2012. Induction of autophagy is essential for monocyte-macrophage differentiation. Blood 119, 2895–2905.
Zhao, Y., Wang, L., Yang, J., et al., 2010. Anti-neoplastic activity of the cytosolic FoxO1 results from autophagic cell death. Autophagy 6, 988–990.
Zhuang, W., Li, B., Long, L., et al., 2011. Knockdown of the DNA-dependent protein kinase catalytic subunit radiosensitizes glioma-initiating cells by inducing autophagy. Brain Res. 1371, 7–15.

CHAPTER

14

The Role of Autophagy in Cancer and Chemotherapy

He Liu, Zhaoyue He and Hans-Uwe Simon

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Abstract

Resistance to current chemo- and radiation therapy is the principal problem in anticancer treatment. Although intensively investigated, the therapeutic outcome is still far from satisfactory. Among the multiple factors which contribute to the drug resistance in cancer cells, the involvement of autophagy is becoming more and more evident. Autophagy describes a cellular self-digestion process, in which cytoplasmic elements can be selectively engulfed and finally degraded in autophagolysosomes to supply nutrients and building blocks for the cells. Autophagy controls cellular homeostasis and can be induced in response to stresses, like hypoxia and growth factor withdrawal. Since the essential physiological function of autophagy is to maintain cellular metabolic balance, dysregulated autophagy has been found associated with multiple diseases, including cancer. Interestingly, the role of autophagy in cancer is two-sided; it can be pro- or antitumor. Autophagy can suppress tumor formation, for example, by controlling cell proliferation and the production of reactive oxygen species. On the other hand, autophagy can provide nutrients to the tumor cells to support tumor growth under nutrition-limiting conditions, thereby promoting tumor development. This ambivalent behavior is also evident in anticancer therapy: By inducing autophagic cell death, autophagy has been shown to potentiate the cytotoxicity of chemotherapeutic drugs, but autophagy has also been linked to drug resistance, since inhibiting autophagy has been found to sensitize tumor cells toward anticancer drug-induced cell death. In this chapter, we will focus on the dual role of autophagy in tumorigenesis and chemotherapy, will classify autophagy inducers and inhibitors used in anticancer treatment, and will discuss topics related to future drug development which have arisen.

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INTRODUCTION

Although maintained at a low level, autophagy is found in all types of eukaryotic cells and can be induced upon nutrient deprivation, growth factor withdrawal, or hypoxia (Liu and Levin, 2014). Supplied by the outer membrane of mitochondria and endoplasmic reticulum, a pre-autophagosomal structure or phagophore is formed to engulf misfolded proteins and damages organelles (Hailey et al., 2010). This pre-autophagosomal structure will be elongated and finally fully sealed, thus forming a double membrane structure, called the autophagosome which then fuses with a lysosome to allow degradation of the enclosed contents by lysosomal enzymes (Choi et al., 2013). Based on the cargos engulfed and degraded and on the involvement of lysosomes, autophagy can be divided into macro- and microautophagy as well as chaperone-mediated autophagy (Hayashi-Nishino et al., 2009, 2010). Unlike macroautophagy and chaperon-mediated autophagy, microautophagy describes the direct engulfment and digestion of cargos by lysosomes (Sahu et al., 2011). In contrast to macro- and microautophagy, which sequester cellular contents, for example, organelles or aggregated cytoplasmic proteins, chaperon-mediated autophagy only engulfs soluble, long-lived proteins (Hayashi-Nishino et al., 2009, 2010). In this review we will focus on macroautophagy, hereafter autophagy.

Induced by a variety of stresses, autophagy promotes cell survival by providing nutrients and building blocks to the cells (Kim et al., 2002; Mizushima and Komatsu, 2011). The autophagy-related proteins, the so-called ATGs, the mammalian target of rapamycin complex 1 (mTORC1), and the class I and III phosphoinositide 3-kinases (PI3Ks) are the main regulators of this dynamic process. mTORC1 and the class I PI3K inhibit autophagy whereas the class III PI3K participates in the activation of autophagy (Shintani and Klionsky, 2004). However, excessive levels of autophagy have been found to be associated with the so called "autophagic cell death" in which the typical double membrane autophagosomes can be observed in the dying cells (Yousefi and Simon, 2009). An answer to the question, whether cells die "by" or "with" autophagy remains elusive. Dysregulated autophagy has been shown to be associated with numerous diseases, including cancer. However, emerging evidence indicates a dual role for autophagy, which can either act pro- or antitumor, that with respect to both tumorigenesis and anticancer therapy (Choi et al., 2013; Guo et al., 2013).

PRO- AND ANTITUMOR FUNCTIONS OF AUTOPHAGY IN TUMORIGENESIS

The tumor-suppressive function of autophagy has been shown in diverse types of cancers both *in vitro* and *in vivo*. Beclin 1 was the first ATG which was shown to suppress tumor formation in breast cancer (Liang et al., 1999; Qu et al., 2003). Ultraviolet radiation resistance–associated gene protein (UVRAG) and endophilin B1 (BIF1), the two components that activate the Beclin 1–class III PI3K complex to induce autophagy, suppress proliferation and tumorigenicity of human colon cancer cells (Liang et al., 2006; Takahashi et al., 2007; Maiuri et al., 2009). ATG5, one of the key players in the formation of autophagosome, has been shown to be downregulated in primary melanoma patients. Furthermore, a deficiency

in ATG5 precludes oncogene-induced senescence in melanocytes, thus contributing to early-stage melanoma carcinogenesis (Liu et al., 2013, 2014). Additionally, *Atg5* disruption was found to promote liver and lung tumorigenesis in mouse models with tissue-specific knockout of *Atg5* (Takamura et al., 2011; Rao et al., 2014). Interestingly, both ATG5 and ATG7 can impose a reciprocal regulation on p53, suggesting that autophagy exhibits feedback mechanisms on p53 controlled molecular pathways (Simon et al., 2014).

On the other hand, a deficiency in autophagy has been shown to reduce tumor development in murine tumor models, underlining the fact of a tumor-promoting function of autophagy in cancer. For instance, conditional knockout of the focal adhesion kinase family-interacting protein of 200 kDa (FIP200), an essential protein required for autophagy initiation, reduced tumorigenesis and subsequent metastasis in an oncogene-driven breast cancer mouse model, thus prolonging survival (Wei et al., 2011). In this study, an elevated infiltration of effector T cells through increased chemokine production was observed in *FIP200* conditional knockout mice, suggesting that inhibition of autophagy by *FIP200* gene disruption may enhance antitumor immune response, thus suppressing mammary tumorigenesis and progression (Wei et al., 2011). Moreover, a recent publication revealed that inhibition of Beclin 1 reduced the partner and localizer of BRCA2 (PALB2)-associated mammary tumorigenesis in a p53 wild type, but not in a p53-null background (Huo et al., 2013). It appeared that autophagy had already been activated by DNA damage and the oxidative stresses induced by the loss of *PALB2*, to mediate tumor cell survival in collaboration with p53, since allelic loss of *Beclin 1* did not affect tumor formation when *p53* was also deleted in the mammary gland (Huo et al., 2013). In this case, autophagy induced by DNA damage responses seemed to ensure tumor cell survival in collaboration with p53.

With an understanding of the role of autophagy in carcinogenesis, research interest in autophagy has become more and more evident. When "autophagy" was used as a search term in PubMed, 3832 publications appeared for the year 2014. When "autophagy and cancer" or "autophagy and cancer therapy" are used as search terms, respectively, 1278 and 398 publications will be listed (Figure 14.1). Parallel to the dual role of autophagy in tumorigenesis, an ambivalence in the role of autophagy in anticancer therapy is also obvious and widely recognized. Although controversial, whether it is a beneficial or an impediment to cancer therapy, autophagy has been recognized as an active player with cancer cells receiving chemotherapeutic drugs.

THE DUAL EFFECT OF AUTOPHAGY IN CHEMOTHERAPY

As the physiological function of autophagy is to protect cells from unfavorable conditions and anticancer therapy is one of the harshest conditions cancer cells can confront, autophagy is often induced upon treatment with diverse antineoplastic drugs. Thus, cancer cells utilize autophagy as a tool to adapt to stressful conditions and to ensure the nutrient supply by autophagic degradation of cellular contents. Although induced, the function of autophagy seems to be ambivalent, on the one hand, enhancing the efficacy of anticancer drugs by inducing the so called "autophagic cell death" and, on the other hand, reducing the cytotoxicity of these drugs, contributing to drug resistance.

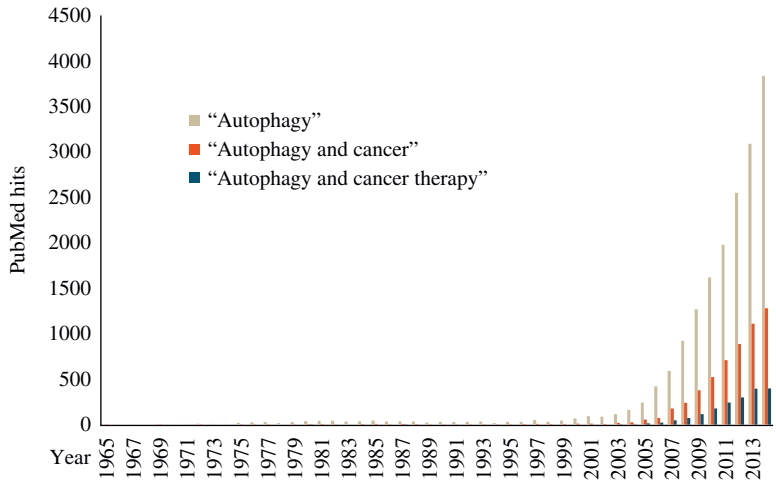

FIGURE 14.1 The exponential increase in autophagy-related publications in PubMed. Hits were plotted against the year searched.

Autophagy Inducers in Chemotherapy

Beclin 1 is deleted in approximately 50% of sporadic breast cancers and enforced *Beclin 1* expression in breast cancer cells reduced proliferation and clonogenicity *in vitro* and *in vivo* (Negri et al., 2010). Similarly, enforced *Atg5* expression in cancer cells resulted in cell death in combination with either chemotherapy (Yousefi et al., 2006) or pharmacological inhibition of autophagy (Maskey et al., 2013). Such findings promoted research on autophagy-inducing agents in cancers. For instance, the antitumor capacity of rapamycin, an mTOR inhibitor, which induces autophagy (Choi et al., 2013), has been reported for a broad spectrum of malignancies, including breast cancer. However, monotherapy using rapamycin or its analogs, such as temsirolimus and everolimus, in different clinical trials ranging from renal cell carcinomas, mantle cell lymphomas, non-resectable neuroendocrine pancreatic tumors as well as advanced breast carcinomas (Kapoor and Figlin, 2009; Krueger et al., 2010; Yao et al., 2011; Baselga et al., 2012) was not as promising as initially expected. A recent study identified a conserved sequence within the Beclin 1 protein which interacts with the virulence factor of the human immunodeficiency virus-1, negative regulatory factor (Pawlak and Dikeakos, 2015), and demonstrated that a cell-permeable peptide was able to induce an antiviral effect in mice (Shoji-Kawata et al., 2013). This peptide is even more potent and specific for inducing autophagy and could be an attractive alternative in anticancer therapy where the induction of autophagy is desired.

Noncanonical autophagy has been reported to contribute to autophagic cell death in breast cancer cells, where autophagy is induced in a Beclin 1-independent manner by resveratrol, a polyphenol found in grapes and peanuts (Scarlatti et al., 2008). Moreover, it has been shown that cannabidiol suppressed the survival of breast cancer cells through induction of both apoptosis and autophagic cell death, this implicating a possible synergetic cell killing effect of both types of cell death (Shrivastava et al., 2011).

Specific kinase inhibitors, such as imatinib and dasatinib, which target the fusion protein between breakpoint cluster region protein and the tyrosine–protein kinase ABL, and also the Akt inhibitor, perifosine, all exhibit antitumor effects in a mono- or combinational therapy in chronic myeloid leukemia (Can et al., 2011), ovarian cancer (Le et al., 2010), or myeloma (Cirstea et al., 2010). Interestingly, metformin, an antidiabetic drug has also been shown to be able to activate AMP-activated protein kinase and induce autophagy, thus suppressing melanoma tumor progression (Tomic et al., 2011). In addition, metformin treatment led to a reduced proliferation and cell cycle arrest in endometrial cancer cells. Inhibition of autophagy by knocking down *Beclin 1* or pharmacologically blocking with 3-methyladenine (3-MA) reduced the antiproliferative effects of metformin on these cells, suggesting that autophagy is indispensable for the metformin-mediated antitumor effect (Takahashi et al., 2014). A similar effect of metformin was also observed in esophageal squamous cell carcinoma in which metformin-induced autophagy and apoptosis by inhibiting the signaling transducer and activator of transcription 3 and B-cell lymphoma 2 (Bcl-2) signaling (Feng et al., 2014).

It is well known that epigenetic alterations, such as DNA methylation and histone modifications, in addition to genetic abnormalities, play an important role in carcinogenesis. Histone deacetylases (HDACs) which are able to remove the acetyl groups of both histones and nonhistone proteins have broad biological functions, ranging from transcriptional regulation to cell cycle control and to autophagy (Li and Zhu, 2014). One of the main mechanisms responsible for HDAC inhibitor (HDACi)–induced autophagy was found to be the mTOR repression in diverse tumor models (Ocker and Schneider-Stock, 2007; Hara et al., 2006; Ohsumi, 2001; Lin et al., 2012). Under normal conditions, mTOR phosphorylates Unc-51-like kinase 1 (ULK1) complex, the autophagy activating components of autophagy, thus inhibiting autophagy (Choi et al., 2013). When mTOR is inactivated by HDACis, ULK1 is released from mTOR suppression and thereby is able to initiate autophagy. It has been shown that suberoylanilide hydroxamic acid (SAHA), a potent HDACi, was able to induce autophagic cell death in endometrial stromal sarcoma cells (Hrzenjak et al., 2008). In addition, in Jurkat cells, autophagy can be induced by SAHA through production of reactive oxygen species (ROS) (Li et al., 2010). On the other hand, induction of autophagy by HDACi can also be cytoprotective. In neuroblastoma cells, genetic or pharmacological inhibition of HDAC 10 sensitized to doxorubicin-mediated cytotoxicity by inhibition of autophagy, indicating that the induction of autophagy can also enhance drug resistance in certain circumstances (Oehme et al., 2013).

Given that Beclin 1 interacts with Bcl-2 through the Bcl homology-3 (BH3) domain, Bcl-2 inhibitors targeting the BH3 domain represent a class of autophagy inducers by releasing Beclin 1 from Bcl-2 binding (Dai et al., 2013). Indeed, (-)-gossypol, a natural BH-3 mimetic, showed a promising anticancer effect in an androgen-independent prostate cancer model overexpressing Bcl-2 by blocking the interaction between Beclin 1 and Bcl-2 to induce autophagy (Lian et al., 2011). Interestingly, a protective autophagy induced by apogossypolone (ApoG2), a derivative of gossypol, has also been observed in hepatocellular carcinoma (HCC) cells, where ApoG2 acted to impeded the interaction of Beclin 1 and Bcl-2, thus inducing ROS-dependent autophagy (Cheng et al., 2013). Inhibition of ROS production by the antioxidant *N*-acetyl-cysteine inhibited autophagy while enhancing ApoG2-induced apoptosis and cell killing in these cells, suggesting a protective role of autophagy in this scenario (Cheng et al., 2013). Proteasome inhibitors, such as carfilzomib and bortezomib, which are able to induce autophagy have been shown to exhibit antitumor effects on non-small-cell lung cancer

cells (Baker et al., 2014) as well as head and neck squamous cell carcinoma cells (Korolchuk et al., 2010) and cervical carcinoma cells (Li and Johnson, 2012).

Taken together, since apoptosis pathways are often dysregulated in cancer cells leading to resistance against most of the anticancer drugs which target apoptosis, induction of autophagic cell death in cancer cells with a mono- or combination therapy to potentiate the efficacy of current chemotherapeutic drugs would seem to be a promising alternative in the rational design of anticancer treatment. Although far from complete, Table 14.1 presents a list of selective autophagy inducers currently being investigated in clinical trials.

Autophagy Inhibitors in Chemotherapy

The primary function of autophagy is to ensure cellular homeostasis under stressful conditions, such as hypoxia and chemo- and radiation therapies (Liu et al., 2013). It is conceivable that autophagy is induced upon treatment of anticancer drugs to protect tumor cells from threatening DNA damage induced by chemotherapeutic drugs. Indeed, emerging evidence indicates that using autophagy inhibitors, either alone or in combination with common chemotherapeutic drugs, increased tumor cell death. These inhibitors inhibit autophagy either by blocking the initiation of autophagy, or by targeting the fusion of the autophagosome with the lysosome, thus inhibiting autophagic flux (Liu et al., 2013). A collection of autophagy inhibitors which have been used in clinical trials is listed in Table 14.1.

3-MA is a well-known class III PI3K inhibitor, which is widely used to inhibit autophagy (Levine et al., 2011) and has been associated with a potentiating antitumor effect for certain chemotherapeutic drugs in several types of cancers (Ding et al., 2011; Li et al., 2009; Bao et al., 2015; Rahmani et al., 2012). Multiple anticancer drugs, such as 5-fluouracil, cisplatin, oxaliplatin, and sorafenib, have been shown to induce a protective autophagy in diverse types of solid tumors, such as HCC and colorectal cancer as well as hematopoietic malignancies (Ding et al., 2011; Li et al., 2009; Bao et al., 2015; Rahmani et al., 2012). Using 3-MA, which blocks at an early stage of autophagy, that is, the formation of autophagosome, the cytotoxicity of these DNA-damaging drugs was significantly increased in, for example, HepG2 cells, a HCC cell line, by augmenting ROS production, which is the major contributor to platinum compound–induced cell death (Ding et al., 2011). In colon cancer cells, autophagy inhibition by 3-MA enhanced 5-FU-mediated apoptosis as evidenced by increased cytochrome c release and caspase-3 activation (Li et al., 2009). A recent study revealed that autophagy induction could be at least one of the mechanisms leading to cisplatin resistance of ovarian cancer. Treatment of human acute myeloid leukemia cells with sorafenib triggered apoptosis accompanied by the induction of autophagy as evidenced by LC3 lipidation and autophagosome formation. Multiple autophagy inhibitors, including 3-MA, were able to enhance the pro-apoptotic effect of sorafenib, suggesting a cytoprotection by autophagy in these cells (Rahmani et al., 2012).

Bafilomycin A1, a H(+)-ATPase inhibitor, which blocks the fusion of the autophagosome with the lysosome to inhibit autophagy (Shacka et al., 2006), has been shown to induce apoptosis and cell cycle arrest in colon cancer cells accompanied with downregulation of cyclin D and E, upregulation of p21 as well as caspase activation (Wu et al., 2009). In human melanoma cells, sanguilutine-induced necroptosis together with autophagy were demonstrated by autophagosome formation and accumulation. Treatment of melanoma cells with

TABLE 14.1 Selective Autophagy Inhibitors and Inducers Used in Clinical Trials

Drugs	Conditions	Phases	NCT Number
AUTOPHAGY INDUCER			
Enzalutamide; Metformin Hydrochloride	Prostate cancer	I	NCT02339168
AUTOPHAGY INHIBITORS			
5-Fluorouracil; Bevacizumab; Hydroxychloroquine; Leucovorin; Oxaliplatin	Rectal cancer; colon cancer; metastasis; adenocarcinoma	I, II	NCT01206530
Abiraterone; ABT-263; Hydroxychloroquine	Prostate cancer	II	NCT01828476
Abraxane; Gemcitabine; Hydroxychloroquine	Pancreatic cancer	II	NCT01978184
Bevacizumab; Carboplatin; Hydroxychloroquine; Paclitaxel	Non-small-cell lung cancer	I, II	NCT00933803
Bevacizumab; Carboplatin; Hydroxychloroquine; Paclitaxel	Non-small-cell lung cancer	II	NCT01649947
Bevacizumab; Carboplatin; Hydroxychloroquine; Paclitaxel	Lung cancer	I, II	NCT00728845
Bevacizumab; Hydroxychloroquine; XELOX regimen	Colorectal cancer	II	NCT01006369
Bortezomib; Hydroxychloroquine	Multiple myeloma and plasma cell neoplasm	I, II	NCT00568880
Cyclophosphamide; Hydroxychloroquine; Velcade	Multiple myeloma	II	NCT01438177
Docetaxel; Hydroxychloroquine	Prostate cancer	II	NCT00786682
Gemcitabine; Hydroxychloroquine	Advanced adenocarcinoma; metastatic adenocarcinoma	I, II	NCT01506973
Gemcitabine; Hydroxychloroquine	Pancreatic cancer	I, II	NCT01128296
Hydroxychloroquine	Breast cancer	II	NCT01292408
Hydroxychloroquine	Melanoma (skin)	0	NCT00962845
Hydroxychloroquine	Small-cell lung cancer	I	NCT00969306
Hydroxychloroquine	Renal cell carcinoma	I	NCT01144169
Hydroxychloroquine	Carcinoma, intraductal, noninfiltrating; DCIS; ductal carcinoma *in situ*	I, II	NCT01023477
Hydroxychloroquine	Prostate cancer	II	NCT00726596
Hydroxychloroquine	Brain metastasis	II	NCT01894633
Hydroxychloroquine	Breast cancer	II	NCT02333890
Hydroxychloroquine; Ixabepilone	Breast cancer	I, II	NCT00765765
Hydroxychloroquine; MK2206	Adult solid neoplasm; prostate cancer; melanoma; renal cell carcinoma	I	NCT01480154

(Continued)

TABLE 14.1 (Continued)

Drugs	Conditions	Phases	NCT Number
Hydroxychloroquine; RAD001	Metastatic clear cell renal cell carcinoma	I, II	NCT01510119
Hydroxychloroquine; Regorafenib; Vorinostat	Colorectal cancer	II	NCT02316340
Hydroxychloroquine; Sorafenib	Refractory or relapsed solid tumors	I	NCT01634893
Hydroxychloroquine; Sunitinib Malate	Adult solid neoplasm	I	NCT00813423
Hydroxychloroquine; Vorinostat	Malignant solid tumor	I	NCT01023737
Hydroxylchloroquine; Trametinib	Advanced BRAF mutant melanoma	I, II	NCT02257424
AUTOPHAGY INDUCERS AND INHIBITORS			
Hydroxychloroquine; Temsirolimus	Unspecified adult solid tumor, protocol specific	I	NCT00909831
Hydroxychloroquine; Sirolimus; Vorinostat	Advanced cancers	I	NCT01266057

https://www.clinicaltrials.gov/ct2/home (as of February 2015).

bafilomycin A1 blocked autophagy and decreased cell viability (Hammerová et al., 2012). An interesting study on the antitumor effect of bafilomycin A1 on B-cell acute lymphoblastic leukemia revealed that low dose of bafilomycin A1 (1 nM) blocked autophagy at both early and late stages by disassociating the Beclin 1–Vps34 complex to induce the binding of Beclin 1 to Bcl-2, thus inhibiting the formation of autophagolysosomes (Yuan et al., 2014).

A large mass of data showing that autophagy inhibition alone or in combination with anticancer drugs achieves an optimized cell killing effect came from the utilization of autophagy inhibitor, chloroquine (CQ) and its analog hydroxylchloroquine (HCQ). In melanoma cells, CQ has been shown to promote apoptosis by stabilizing the P53 upregulated modulator of apoptosis, an effect that has not been observed with other lysosomal pH-affecting agents, such as bafilomycin A1 and cathepsin inhibitors (Lakhter et al., 2013). Interestingly, in the mouse breast cancer cell lines, HCQ seemed also to enhance cisplatin-induced cell death that is not dependent on autophagy, since autophagy inhibition, for example, by knocking down *Atg12* or *Beclin 1* or by treatment of cells with bafilomycin A1, did not sensitize these cells toward cisplatin-induced cell killing (Maycotte et al., 2012). In a Myc-induced lymphoma which is resistant to apoptosis due to the lack of p53, inhibition of autophagy by CQ-induced tumor cell death (Amaravadi et al., 2007). Another study in glioblastoma showed that CQ was able to induce cell death in five different glioblastoma cells with different p53 gene mutation status. Caspase inhibitors could not attenuate cell death in these cells treated with CQ, although caspase-3 activation was observed. In all the cell lines, an accumulation of autophagosomes was apparent and the levels and distribution of cathepsin D were altered, indicating that lysosomal dysfunction may be involved in CQ-induced cell death in these cells (Geng et al., 2010).

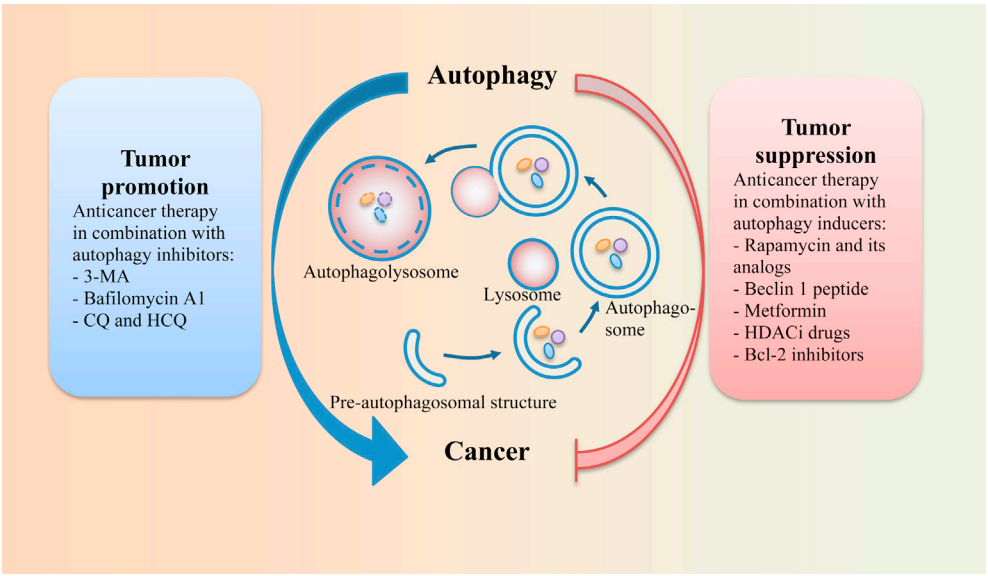

FIGURE 14.2 The dual role of autophagy in cancer and the corresponding application of autophagy-modulating compounds in anticancer therapy. As a cellular stress–responsive mechanism, autophagy can be either pro- or anticancer. Accordingly, both autophagy inducers and inhibitors can be used to sensitize tumor cells toward chemotherapeutic drugs.

Compared to the pharmacological inhibition of autophagy, genetic modulation of autophagy by knocking down essential *ATGs* seemed to be more specific for targeting the protective autophagy induced by chemotherapy. Indeed, a large body of data obtained from, for example, melanoma cells treated with diverse types of antineoplastic drugs, showed that the inhibition of autophagy by knocking down *ATG5* or *Beclin 1* had a synergetic cell killing effect (Marino et al., 2010; Tomic et al., 2011).

DISCUSSION

Given that the majority of cancer cells are defective in apoptosis pathways, anticancer drugs targeting the apoptotic signaling networks often lead to drug resistance in tumor cells. Modulating alternative pathways, such as autophagy, would seem to be a rational goal for design of future anticancer therapies. Indeed, evidence obtained from basic and clinical research indicates the basal level of autophagy is often abnormal, either up- or downregulated in different types of tumors. Upon treatment of cancer cells with antineoplastic agents, autophagy is often upregulated, in the consequence either promoting or inhibiting therapy-mediated cell killing. Although controversial, autophagy has opened up to us a new avenue for finding more promising tools for fighting cancer. However, caution is advised based on the large body of evidence showing that autophagy can promote pro- as well as anti-cancer effects, both during tumorigenesis and anticancer therapy (Figure 14.2).

The major obstacle for making therapy decisions about targeting autophagy is measuring or evaluating the level of autophagy in the tumor cells of a cancer patient *in vivo*. Unlike tumor cells in culture dishes, where the basal level of autophagy and the autophagic response of these tumor cells can be assessed by monitoring the dynamic formation of autophagosomes, for example, by detecting LC3 lipidation using immunoblotting or by monitoring autophagosome formation using confocal or electron microscopy, measuring autophagy in tumor cells in patients is still challenging. Given that autophagy is a dynamic, rather than a static process, increased autophagosome formation does not necessarily mean an induction of autophagy, since merely blocking the degradation of engulfed contents could also lead to an increase in autophagosomes. For this reason, autophagy inhibitors, such as HCQ and bafilomycin A1, are often used to exclude this latter case. However, such an approach is not feasible in a living organism, so that monitoring the level of p62 which is a specific substrate of autophagic degradation remains the indispensable standard. Unfortunately, p62 has been shown to be transcriptionally regulated by diverse stimuli, especially ROS (Bjørkøy et al., 2009). Therefore, robust controls must be included when an immunohistochemical staining of p62 is employed for evaluating the level of autophagy in a patient. Accordingly, it is difficult to conclude whether an effective therapy is due to a successful modulation of dysregulated autophagy in cancer patients, since methods to evaluate the level of autophagy before and after therapy are limited.

Another problem is the lack of specificity among the autophagy-modulating pharmacological agents. Rapamycin and its analogs, HDACis as well as the most widely used autophagy inhibitors, CQ and HCQ, have broad biological functions rather than simply controlling autophagy. For example, HCQ is a widely used antimalarial drug because of its relative safety and low cost. For the same reason, HCQ is one of the most commonly used autophagy-targeting drugs in clinical trials (Table 14.1). However, it has been shown that the sensitization to an anticancer drug in tumor cells can occur in both an autophagy-dependent and an autophagy-independent manner (Maycotte et al., 2012). In addition, the effectiveness of HCQ as an adjunct in chemo- and radiation sensitization was variable depending on the drug/tumor models (Bristol et al., 2013). In the murine 4T1 breast cancer cells, even knocking down an essential *ATG*, *Atg12*, did not affect the clonogenicity of these cells as compared with radiation alone (Bristol et al., 2013), emphasizing once again the cancer type–dependent feature of autophagy. Associated to the questionable specificity of these inducers and inhibitors of autophagy, these drugs are surely not only targeting tumor cells, which may in the first place exhibit abnormal levels of autophagy, but they also modify autophagy in normal cells.

Taken together, findings to date suggest that modulating autophagy might be promising alternative/supplement to current mono- and combination therapies and have an intense interest in basic research and clinical studies. However, the answers to basic questions, such as *whether* all types of cancers have dysregulated autophagy and *how* to measure the level of autophagy in patients, still remain unclear. These answers are essential for improving our current understanding of the role of autophagy in cancer and its potential for anticancer therapy.

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References

Amaravadi, R.K., Yu, D., Lum, J.J., et al., 2007. Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. J. Clin. Invest. 117, 326–336.

Baker, A.F., Hanke, N.T., Sands, B.J., et al., 2014. Carfilzomib demonstrates broad anti-tumor activity in pre-clinical non-small cell and small cell lung cancer models. J. Exp. Clin. Cancer Res. 33, 111.

Bao, L., Jaramillo, M.C., Zhang, Z., et al., 2015. Induction of autophagy contributes to cisplatin resistance in human ovarian cancer cells. Mol. Med. Rep. 11, 91–98.

Baselga, J., Campone, M., Piccart, M., et al., 2012. Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. N. Engl. J. Med. 366, 520–529.

Bjørkøy, G., Lamark, T., Pankiv, S., et al., 2009. Monitoring autophagic degradation of p62/SQSTM1. Methods Enzymol. 452, 181–197.

Bristol, M.L., Emery, S.M., Maycotte, P., et al., 2013. Autophagy inhibition for chemosensitization and radiosensitization in cancer: do the preclinical data support this therapeutic strategy? J. Pharmacol. Exp. Ther. 344, 544–552.

Can, G., Ekiz, H.A., Baran, Y., 2011. Imatinib induces autophagy through BECLIN-1 and ATG5 genes in chronic myeloid leukemia cells. Hematology 16, 95–99.

Cheng, P., Ni, Z., Dai, X., et al., 2013. The novel BH-3 mimetic apogossypolone induces Beclin-1- and ROS-mediated autophagy in human hepatocellular carcinoma [corrected] cells. Cell Death Dis. 4, e489.

Choi, A.M., Ryter, S.W., Levine, B., 2013. Autophagy in human health and disease. N. Engl. J. Med. 368, 651–662.

Cirstea, D., Hideshima, T., Rodig, S., et al., 2010. Dual inhibition of akt/mammalian target of rapamycin pathway by nanoparticle albumin-bound-rapamycin and perifosine induces antitumor activity in multiple myeloma. Mol. Cancer Ther. 9, 963–975.

Dai, J.P., Zhao, X.F., Zeng, J., et al., 2013. Drug screening for autophagy inhibitors based on the dissociation of Beclin1-Bcl2 complex using BiFC technique and mechanism of eugenol on anti-influenza A virus activity. PLoS One 8, e61026.

Ding, Z.B., Hui, B., Shi, Y.H., et al., 2011. Autophagy activation in hepatocellular carcinoma contributes to the tolerance of oxaliplatin via reactive oxygen species modulation. Clin. Cancer Res. 17, 6229–6238.

Feng, Y., Ke, C., Tang, Q., et al., 2014. Metformin promotes autophagy and apoptosis in esophageal squamous cell carcinoma by downregulating Stat3 signaling. Cell Death Dis. 5, e1088.

Geng, Y., Kohli, L., Klocke, B.J., et al., 2010. Chloroquine-induced autophagic vacuole accumulation and cell death in glioma cells is p53 independent. Neuro Oncol. 12, 473–481.

Guo, J.Y., Xia, B., White, E., 2013. Autophagy-mediated tumor promotion. Cell 155, 1216–1219.

Hailey, D.W., Rambold, A.S., Satpute-Krishnan, P., et al., 2010. Mitochondria supply membranes for autophagosome biogenesis during starvation. Cell 141, 656–667.

Hammerová, J., Uldrijan, S., Táborská, E., et al., 2012. Necroptosis modulated by autophagy is a predominant form of melanoma cell death induced by sanguilutine. Biol. Chem. 393, 647–658.

Hara, T., Nakamura, K., Matsui, M., et al., 2006. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature 441, 885–889.

Hayashi-Nishino, M., Fujita, N., Noda, T., et al., 2009. A subdomain of the endoplasmic reticulum forms a cradle for autophagosome formation. Nat. Cell Biol. 11, 1433–1437.

Hayashi-Nishino, M., Fujita, N., Noda, T., et al., 2010. Electron tomography reveals the endoplasmic reticulum as a membrane source for autophagosome formation. Autophagy 6, 301–303.

Hrzenjak, A., Kremser, M.L., Strohmeier, B., et al., 2008. SAHA induces caspase-independent, autophagic cell death of endometrial stromal sarcoma cells by influencing the mTOR pathway. J. Pathol. 216, 495–504.

Huo, Y., Cai, H., Teplova, I., et al., 2013. Autophagy opposes p53-mediated tumor barrier to facilitate tumorigenesis in a model of PALB2-associated hereditary breast cancer. Cancer Discov. 3, 894–907.

Kapoor, A., Figlin, R.A., 2009. Targeted inhibition of mammalian target of rapamycin for the treatment of advanced renal cell carcinoma. Cancer 115, 3618–3630.

Kim, J., Huang, W.P., Stromhaug, P.E., et al., 2002. Convergence of multiple autophagy and cytoplasm to vacuole targeting components to a perivacuolar membrane compartment prior to *de novo* vesicle formation. J. Biol. Chem. 277, 763–773.

Korolchuk, V.I., Menzies, F.M., Rubinsztein, D.C., 2010. Mechanisms of cross-talk between the ubiquitin-proteasome and autophagy-lysosome systems. FEBS Lett. 584, 1393–1398.

Krueger, D.A., Care, M.M., Holland, K., et al., 2010. Everolimus for subependymal giant-cell astrocytomas in tuberous sclerosis. N. Engl. J. Med. 363, 1801–1811.
Lakhter, A.J., Sahu, R.P., Sun, Y., et al., 2013. Chloroquine promotes apoptosis in melanoma cells by inhibiting BH3 domain-mediated PUMA degradation. J. Invest. Dermatol. 133, 2247–2254.
Le, X.F., Mao, W., Lu, Z., et al., 2010. Dasatinib induces autophagic cell death in human ovarian cancer. Cancer 116, 4980–4990.
Levine, B., Mizushima, N., Virgin, H.W., 2011. Autophagy in immunity and inflammation. Nature 469, 323–335.
Li, C., Johnson, D.E., 2012. Bortezomib induces autophagy in head and neck squamous cell carcinoma cells via JNK activation. Cancer Lett. 314, 102–107.
Li, J., Hou, N., Faried, A., et al., 2009. Inhibition of autophagy by 3-MA enhances the effect of 5-FU-induced apoptosis in colon cancer cells. Ann. Surg. Oncol. 16, 761–771.
Li, J., Liu, R., Lei, Y., et al., 2010. Proteomic analysis revealed association of aberrant ROS signaling with suberoylanilide hydroxamic acid-induced autophagy in Jurkat T-leukemia cells. Autophagy 6, 711–724.
Li, Z., Zhu, W.G., 2014. Targeting histone deacetylases for cancer therapy: from molecular mechanisms to clinical implications. Int. J. Biol. Sci. 10, 757–770.
Lian, J., Wu, X., He, F., et al., 2011. A natural BH3 mimetic induces autophagy in apoptosis-resistant prostate cancer via modulating Bcl-2-Beclin1 interaction at endoplasmic reticulum. Cell Death Differ. 18, 60–71.
Liang, C., Feng, P., Ku, B., et al., 2006. Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG. Nat. Cell Biol. 8, 688–699.
Liang, X.H., Jackson, S., Seaman, M., et al., 1999. Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature 402, 672–676.
Lin, S.Y., Li, T.Y., Liu, Q., et al., 2012. GSK3-TIP60-ULK1 signaling pathway links growth factor deprivation to autophagy. Science 336, 477–481.
Liu, H., He, Z., von Rütte, T., et al., 2013. Down-regulation of autophagy-related protein 5 (ATG5) contributes to the pathogenesis of early-stage cutaneous melanoma. Sci. Transl. Med. 5, 202ra123.
Liu, H., He, Z., Simon, H.U., 2013. Targeting autophagy as a potential therapeutic approach for melanoma therapy. Semin. Cancer Biol. 23, 352–360.
Liu, H., He, Z., Simon, H.U., 2014. Autophagy suppresses melanoma tumorigenesis by inducing senescence. Autophagy 10, 372–373.
Liu, Y., Levine, B., 2014. Autosis and autophagic cell death: the dark side of autophagy. Cell Death Differ. 22, 367–376.
Maiuri, M.C., Tasdemir, E., Criollo, A., et al., 2009. Control of autophagy by oncogenes and tumor suppressor genes. Cell Death Differ. 16, 87–93.
Marino, M.L., Fais, S., Djavaheri-Mergny, M., et al., 2010. Proton pump inhibition induces autophagy as a survival mechanism following oxidative stress in human melanoma cells. Cell Death Dis. 1, e87.
Maskey, D., Yousefi, S., Schmid, I., et al., 2013. ATG5 is induced by DNA-damaging agents and promotes mitotic catastrophe independent of autophagy. Nat. Commun. 4, 2130.
Maycotte, P., Aryal, S., Cummings, C.T., et al., 2012. Chloroquine sensitizes breast cancer cells to chemotherapy independent of autophagy. Autophagy 8, 200–212.
Mizushima, N., Komatsu, M., 2011. Autophagy: renovation of cells and tissues. Cell 147, 728–741.
Negri, T., Tarantino, E., Orsenigo, M., et al., 2010. Chromosome band 17q21 in breast cancer: significant association between beclin 1 loss and HER2/NEU amplification. Genes Chromosomes Cancer 49, 901–909.
Ocker, M., Schneider-Stock, R., 2007. Histone deacetylase inhibitors: signalling towards p21cip1/waf1. Int. J. Biochem. Cell Biol. 39, 1367–1374.
Oehme, I., Linke, J.P., Böck, B.C., et al., 2013. Histone deacetylase 10 promotes autophagy-mediated cell survival. Proc. Natl. Acad. Sci. U.S.A. 110, E2592–E2601.
Ohsumi, Y., 2001. Molecular dissection of autophagy: two ubiquitin-like systems. Nat. Rev. Mol. Cell Biol. 2, 211–216.
Pawlak, E.N., Dikeakos, J.D., 2015. HIV-1 Nef: a master manipulator of the membrane trafficking machinery mediating immune evasion. Biochim. Biophys. Acta 1850, 733–741.
Qu, X., Yu, J., Bhagat, G., et al., 2003. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. J. Clin. Invest. 112, 1809–1820.

Rahmani, M., Aust, M.M., Attkisson, E., et al., 2012. Inhibition of Bcl-2 antiapoptotic members by obatoclax potently enhances sorafenib-induced apoptosis in human myeloid leukemia cells through a Bim-dependent process. Blood 119, 6089–6098.

Rao, S., Tortola, L., Perlot, T., et al., 2014. A dual role for autophagy in a murine model of lung cancer. Nat. Commun. 5, 3056.

Sahu, R., Kaushik, S., Clement, C.C., et al., 2011. Microautophagy of cytosolic proteins by late endosomes. Dev. Cell 20, 131–139.

Scarlatti, F., Maffei, R., Beau, I., et al., 2008. Role of non-canonical Beclin 1-independent autophagy in cell death induced by resveratrol in human breast cancer cells. Cell Death Differ. 15, 1318–1329.

Shacka, J.J., Klocke, B.J., Roth, K.A., 2006. Autophagy, bafilomycin and cell death: the "a-B-cs" of plecomacrolide-induced neuroprotection. Autophagy 2, 228–230.

Shintani, T., Klionsky, D.J., 2004. Autophagy in health and disease: a double-edged sword. Science 306, 990–995.

Shoji-Kawata, S., Sumpter, R., Leveno, M., et al., 2013. Identification of a candidate therapeutic autophagy-inducing peptide. Nature 494, 201–206.

Shrivastava, A., Kuzontkoski, P.M., Groopman, J.E., et al., 2011. Cannabidiol induces programmed cell death in breast cancer cells by coordinating the cross-talk between apoptosis and autophagy. Mol. Cancer Ther. 10, 1161–1172.

Simon, H.U., Yousefi, S., Schmid, I., et al., 2014. ATG5 can regulate p53 expression and activation. Cell Death Dis. 5, e1339.

Takahashi, A., Kimura, F., Yamanaka, A., et al., 2014. Metformin impairs growth of endometrial cancer cells via cell cycle arrest and concomitant autophagy and apoptosis. Cancer Cell Int. 14, 53.

Takahashi, Y., Coppola, D., Matsushita, N., et al., 2007. Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. Nat. Cell Biol. 9, 1142–1151.

Takamura, A., Komatsu, M., Hara, T., et al., 2011. Autophagy deficient mice develop multiple liver tumors. Genes Dev. 25, 795–800.

Tomic, T., Botton, T., Cerezo, M., et al., 2011. Metformin inhibits melanoma development through autophagy and apoptosis mechanisms. Cell Death Dis. 2, e199.

Wei, H., Wei, S., Gan, B., et al., 2011. Suppression of autophagy by FIP200 deletion inhibits mammary tumorigenesis. Genes Dev. 25, 1510–1527.

Wu, Y.C., Wu, W.K., Li, Y., et al., 2009. Inhibition of macroautophagy by bafilomycin A1 lowers proliferation and induces apoptosis in colon cancer cells. Biochem. Biophys. Res. Commun. 382, 451–456.

Yao, J.C., Shah, M.H., Ito, T., et al., 2011. RAD001 in Advanced Neuroendocrine Tumors, Third Trial (RADIANT-3) Study Group. Everolimus for advanced pancreatic neuroendocrine tumors. N. Engl. J. Med. 364, 514–523.

Yousefi, S., Simon, H.U., 2009. Autophagy in cancer and chemotherapy. Results Probl. Cell Differ. 49, 183–190.

Yousefi, S., Perozzo, R., Schmid, I., et al., 2006. Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. Nat. Cell Biol. 8, 1124–1132.

Yuan, N., Song, L., Zhang, S., et al., 2014. Bafilomycin A1 targets both autophagy and apoptosis pathways in pediatric B-cell acute lymphoblastic leukemia. Haematologica 100, 345–356.

CHAPTER

15

Autophagy Activation in the Tumor Microenvironment: A Major Process in Shaping the Antitumor Immune Response

Bassam Janji, Elodie Viry, Takouhie Mgrditchian, Tsolere Arakelian, Sandrine Medves and Guy Berchem

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Abstract

The role of autophagy in cancer is complex and is likely dependent on tumor type, stage, and genetic context. This complexity is illustrated by the identification of settings where autophagy acts potently to either promote or inhibit tumorigenesis. It is now well established that autophagy serves as a barrier to limit tumor initiation; however, once tumors are well established, autophagy plays a positive role in malignant progression and in subsequent tumor maintenance. Autophagy is also induced in response to cancer therapies where it can function as a survival mechanism and limit drug efficacy. Such findings have inspired significant interest in applying anti-autophagy therapies as an entirely new approach to cancer treatment. While much remains to be learned about the regulation and context-dependent biological role of autophagy, it is now widely established that modulation of this process will be an attractive avenue for future anticancer therapeutic approaches.

In this chapter, we will summarize recent data describing how autophagy activation under hypoxic stress impairs the antitumor immune response and compromises cancer immunotherapeutic approaches. In addition, we will discuss how tumor manages to hide from the immune attack, and either mounts a "counterattack" or develops resistance to immune cells. In particular, we will focus on the effect of hypoxia-induced autophagy in allowing tumor cells to outmaneuver an effective immune response and escape from immune surveillance. It is our belief that autophagy may represent a conceptual realm for new immunotherapeutic strategies aiming to block immune escape and therefore providing rational approach to future tumor immunotherapy design.

INTRODUCTION

During the past decade it became clear that tumors are highly heterogeneous and should be conceived as organs composed of different tumor cell types and tumor-associated cell types, including fibroblasts, endothelial cells, and immune cells, all together constituting the tumor microenvironment (TME).

Several types of immune cells are involved in tumor immune surveillance. Briefly, key cells of the adaptive immune system identifying cancer cells are the cytotoxic T lymphocytes (CTL) which are able to recognize tumor antigens *via* the T-cell receptor (TCR). These antigens are exclusively expressed by tumors and are called tumor-specific antigens (Smyth et al., 2001). Natural killer (NK) cells of the innate immune system also play an important role in tumor immune surveillance by mechanisms called "missing-self" and "induced-self" recognitions (Watzl and Long, 2000). While the molecular mechanisms by which CTL and NK cells recognize their target tumor cells are fundamentally different, both types of immune cells kill their targets following the establishment of immunological synapse (IS) (Grakoui et al., 1999). The formation of IS requires cell polarization and extensive remodeling of the actin cytoskeleton at various stages (Dustin and Cooper, 2000). It has been reported that autophagy plays a role in the stabilization of the IS between dendritic cells (DCs) and T cells. Indeed, knockdown of autophagy-related protein 16-like 1 (ATG16L1) and immunity-related GTPase family M protein with small interfering RNAs (siRNAs) in DCs resulted in hyperstable interactions between DCs and T cells, increased activation of T cells, and induction of a T-helper 17 cell response. In addition, DCs from patients with Crohn's disease who had an ATG16L1 risk allele had a similar hyperstability of the immunological synapse (Wildenberg et al., 2012). Whether or not autophagy stabilizes the IS between tumor and immune cells remains to be determined.

It is now well established that CTL and NK cells recognize and kill target cells by two major pathways: either through the release of cytotoxic granules containing perforin and

granzymes to the cytosol of target cells (Shresta et al., 1995), or through tumor necrosis factor (TNF) super family-dependent killing (Cullen et al., 2010).

Several lines of evidence indicate that the majority of immune effector cells recruited to the tumor site displayed a reduced cytotoxicity toward tumor cells and therefore their antitumor functions were largely downregulated in response to several microenvironmental factors. Indeed, in the TME, hypoxic stress—the result of an inadequate oxygen supply to the cells and tissues—is a characteristic feature of locally advanced solid tumors and is considered as one of the major mechanisms responsible for tumor resistance to therapies (Harris, 2002).

Experimental and clinical evidence indicates that the majority of mechanisms suppressing the antitumor immune functions directly evolve from the hypoxic TME (reviewed in Chouaib et al., 2014). Thus, NK cells and natural killer T (NKT) cells infiltrate the TME, but are not found in contact with tumor cells (Balkwill et al., 2012). It has been reported that in colorectal, gastric, lung, renal, and liver cancer, NK cells appear to predict a good prognosis (Tachibana et al., 2005). However, although they are present in the TME, NK cells may not be able to exert their tumor-killing function. A number of studies reported that NK cells in the tumor stroma have an anergic phenotype that is induced by malignant cell–derived transforming growth factor-beta (Fridman et al., 2012). Furthermore, immune cells in the TME not only fail to exercise antitumor effector functions, but also are co-opted to promote tumor growth (Whiteside, 2008). In addition, it has become clear that the immune system not only protects the host against tumor development but also sculpts the immunogenic phenotype of a developing tumor and can favor the emergence of resistant tumor cell variants (Hamai et al., 2010).

Several lines of evidence indicate that autophagy is frequently increased in established tumors, and high level of autophagy is often found in hypoxic TME. Recent studies support the concept that advanced tumors display an "autophagy addiction" that is required to maintain their energy balance, through the recycling of intracellular components into biosynthetic pathways or adenosine 5′-triphosphate (ATP) synthesis (Lum et al., 2005; White, 2012). The stimulation of autophagy in the TME can contextually favor the emergence of resistant tumor cell variants and weaken the antitumor immunity and therefore severely compromise the tumor immune surveillance. Given that immune cells in the TME are also exposed to hypoxia, it is likely that they too become autophagic. Whether the activation of autophagy helps or hinders immune cell function remains to be demonstrated. Understanding the physiological consequences of autophagy in different cell types in the TME is critical when considering therapies that target autophagy.

In this chapter, we will describe the molecular mechanisms by which hypoxic stress activates autophagy in tumor cells and the functional consequences of this activation regarding the antitumor immune response mediated by CTL and NK cells.

HYPOXIA-DEPENDENT ACTIVATION OF AUTOPHAGY

In this section, we will describe the mechanisms involved in the induction of autophagy in tumor cells under hypoxic stress.

Hypoxia-inducible factor-1 (HIF-1) is the key transcription factor that allows survival and rapid adaptation of cells to hypoxia. Briefly, HIF-1 is a heterodimer, composed of a

constitutively expressed subunit and an O_2-regulated subunit, HIF-1β and HIF-1α, respectively (Majmundar et al., 2010). In the presence of oxygen, HIF-1α is hydroxylated on a proline residue by prolyl hydroxylase domain protein 2, which leads to an interaction with the von Hippel–Lindau (VHL) protein (Ke and Costa, 2006). This allows the recruitment of an E3 ubiquitin ligase that catalyzes the polyubiquitination of HIF-1α and its subsequent degradation by ubiquitin proteasome system (UPS) (Ke and Costa, 2006). Under hypoxia, the hydroxylation of HIF-1α is inhibited and HIF-1α is accumulated and translocated to the nucleus where it forms a dimer with HIF-1β and activates the transcription of several genes involved in many biological processes including autophagy (Ke and Costa, 2006). It has been described that the severity and duration of hypoxia differently regulate the autophagic pathway. Indeed, in chronic and moderate hypoxia, autophagy can be induced through HIF-1α and protein kinase C delta/c-Jun N-terminal kinase 1 dependent pathways (Mazure and Pouyssegur, 2010). Conversely, during rapid and severe oxygen fluctuation conditions, which are often coupled with energy and glucose restriction, autophagy is induced *via* HIF-independent pathways. These signaling mechanisms include the mammalian target of rapamycin (mTOR) pathway (Papandreou et al., 2008) and unfolded protein response of endoplasmic reticulum stress pathway (Rouschop et al., 2010). Below, we will mainly describe the molecular mechanisms by which autophagy is activated in a HIF-1α-dependent manner.

It has been reported that under hypoxia, autophagy can be induced through HIF-1α-dependent expression of the BH3-only protein Bcl-2/adenovirus E1B 19 kDa-interacting protein 3 (BNIP3) and the related protein, BNIP3L (Mazure and Pouyssegur, 2009). These proteins are downstream targets of HIF-1α and are able to induce also mitophagy to manage reactive oxygen species production. Although the promoter of BNIP3 contains two hypoxia-responsive elements (HREs), HRE1 and HRE2, it has been shown that the induction of BNIP3 occurs through the direct binding of HIF-1α to HRE2 (Kothari et al., 2003). Mechanistically, Bellot et al. (2009) showed that induction of BNIP3 and BNIP3L in hypoxic cells disrupts the Beclin1 (BECN1)/B-cell lymphoma 2 (Bcl-2) complex leading to BECN1 release and the subsequent autophagy induction as an adaptive survival response during prolonged hypoxia (Bellot et al., 2009) (Figure 15.1). We have shown that the acquisition of TNF resistance in breast cancer cells is correlated with constitutive activation of HIF-1α, even under normoxia, and with the induction of autophagy by several signaling pathways highlighting the important role of autophagy in tumor cell adaptation to hypoxia (Moussay et al., 2011).

EFFECTS OF HYPOXIA-INDUCED AUTOPHAGY ON THE ANTITUMOR IMMUNE RESPONSE

Emerging recent data have shown that hypoxia-induced autophagy is an important regulator of the innate and adaptive antitumor immunity mediated by NK cells and CTL, respectively. In particular, hypoxia has been described to play a central role in activating multiple overlapping mechanisms involving autophagy and leading to the emergence of resistant tumor cells able to outmaneuver an effective immune response and escape from immune cell killing (Baginska et al., 2013b). In this context, we have recently shown that the

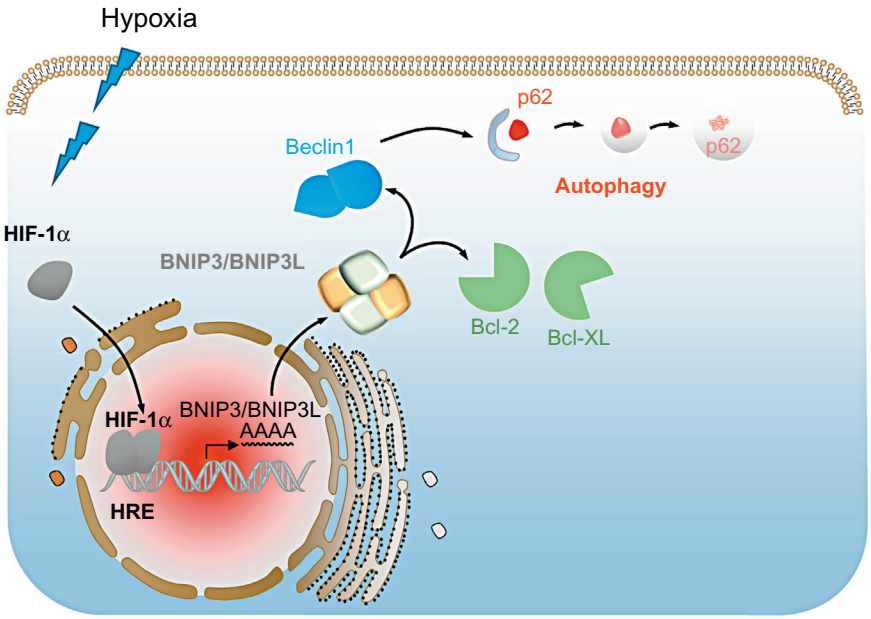

FIGURE 15.1 Molecular mechanism of autophagy induction by hypoxic stress. Under hypoxia, the stabilization of HIF-1α leads to its translocation to the nucleus and a rapid induction of the BH3-only proteins (BNIP3 and BNIP3L) through its binding to the HRE in the promoter of BNIP3. The induction of BNIP3 and BNIP3L displaces Beclin1 from Bcl-XL and Bcl-2, leading to the induction of autophagy.

activation of autophagy in tumor cells under hypoxia dramatically decreases tumor cell susceptibility to NK- and CTL-mediated lysis by multiple mechanisms (Baginska et al., 2013a; Messai et al., 2014; Noman et al., 2011). Therefore, autophagy activation is considered to be an important adaptive and resistance mechanism operating in tumor cells to escape the immune system. In accordance with such a role of autophagy, Lotze et al. (2012) showed that NK cells, along with human peripheral blood lymphocytes, are primary mediators in inducing autophagy in several human tumors, promoting cancer cell survival (Buchser et al., 2012; Liang et al., 2012; Lotze et al., 2012). Other studies showed that autophagy also plays an important role in regulating CTL-mediated antitumor immune response (Noman et al., 2011, 2012). While the molecular mechanisms by which autophagy impairs tumor susceptibility to NK and CTL are different, experimental evidence claims that blocking autophagy may improve antitumor immunity.

Functional Role of Autophagy Induction on T-Cell-Mediated Antitumor Immune Response

Autophagy activation not only enables tumor cells to survive stress conditions during cancer development but also provides the abilities to modulate the antitumor immune response. Michaud et al. (2011) showed that chemotherapy-induced autophagy in mice causes the release of ATP from tumor cells, thereby stimulating antitumor immune

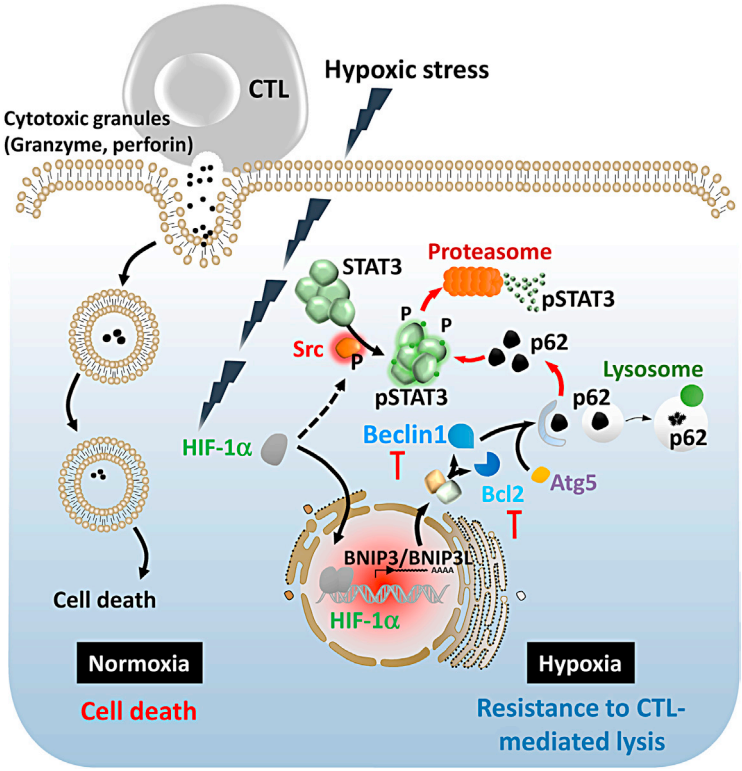

FIGURE 15.2 Regulation of CTL-mediated tumor cell lysis by hypoxia-induced autophagy. Hypoxic stress leads to the accumulation of HIF-1α. By a yet undefined mechanism, HIF-1α increases the level of phospho-Src, which subsequently phosphorylates STAT3 at the Tyr705 residue. As HIF-target gene products, BNIP3 and BNIP3L are transcriptionally upregulated and compete with the BECN1–BCL2 complex. This competition releases BECN1 from the complex and then activates the autophagic machinery by recruiting several autophagic proteins including ATG5. As an autophagic substrate, p62/SQSTM1 is degraded in the autophagosomes following their fusion with lysosomes. In view of the fact that p62/SQSTM1 is involved in targeting pSTAT3 to the UPS, its degradation leads to the accumulation of pSTAT3 in cells. In autophagy-defective cells, p62/SQSTM1 is no longer degraded, and its accumulation accelerates the UPS-dependent degradation of pSTAT3. *Source: Adapted from Noman et al. (2012).*

responses (Michaud et al., 2011). The autophagy-dependent extracellular ATP release recruits DCs into tumors and activates a T-cell response to tumor cells. They demonstrated that targeting ATG5 or ATG7 genes, encoding essential autophagy proteins, in highly immunogenic allograft mouse tumors, blunted the release of ATP by the tumor cells in response to chemotherapy. This study highlights that autophagy, activated in the context of DNA damage-induced apoptosis, causes ATP release and the recruitment of immune cells. Although tumor cell autophagy may recruit the immune system, it can also limit immune-mediated cytotoxicity in the context of hypoxic stress. The first evidence for such a role of autophagy was provided by Noman et al. who demonstrated that hypoxic lung carcinoma cells can evade CTL-mediated lysis through autophagy induction (Noman et al., 2011, 2012) (Figure 15.2). Indeed, we showed that inhibition of autophagy using siRNAs directed against ATG5

or BECN1 restored tumor cells sensibility to CTL-mediated lysis. This was correlated with a decrease in hypoxia-dependent induction of the phosphorylation of signal transducer and activator of transcription 3 (STAT3). These results allowed the prediction that blocking autophagy would suppress phosphoSTAT3 (pSTAT3)-dependent survival mechanism making tumor cells more susceptible to CTL attack under hypoxia. Considering the degradation role of autophagy, it is difficult however to perceive how autophagy is involved in the stabilization of pSTAT3 under hypoxia. Focusing on the cross talk between the adaptor protein sequestosome1 (SQSTM1/p62), UPS, and autophagy, we revealed that the induction of HIF-1α has two effects in tumor cells: (i) HIF-1α triggers the phosphorylation of Src which subsequently phosphorylates the tyrosine residue Y705 of STAT3; (ii) HIF-1α activates autophagy by a mechanism involving the increased expression of BNIP3/BNIP3L and the dissociation of the BECN1/Bcl-2 complex. Autophagy activation results in the degradation of the p62 protein. Knowing that p62 is the receptor/adaptor protein responsible for targeting pSTAT3 to the UPS, the autophagy-dependent degradation of p62 leads to the accumulation of pSTAT3. When autophagy is inhibited in tumor cells, the degradation of p62 is blocked and therefore p62 accumulates in tumor cells. This accumulation accelerates the UPS-dependent degradation of pSTAT3 (Noman et al., 2011, 2012). The effect of the autophagy inhibitor hydroxychloroquine (HCQ) was also evaluated *in vivo* in combination with a tyrosinase-related protein-2 (TRP2) peptide-based vaccination strategy in order to induce T-cell response. Using a transplantable murine melanoma B16-F10 cell line, we provided evidence that autophagy is primarily detected in hypoxic areas of the tumor. Inhibition of autophagy in B16-F10 engrafted tumors resulted in a significant decrease in tumor growth by inducing apoptosis, as revealed by TUNEL staining. These results strongly argue for a role of autophagy in mediating hypoxia tolerance to the immune system. More interestingly, a significant decrease in tumor growth was observed in vaccinated and HCQ-treated group of mice as compared to control and to treatment alone (Figure 15.3). These results strongly suggest that *in vivo* inhibition of autophagy improves the antitumor effect of a TRP2-based vaccine.

Functional Role of Autophagy Induction on NK-Cell-Mediated Tumor Immune Response

NK cells are potent cytolytic lymphocytes belonging to the innate immune system. Although resting NK cells circulate in the blood, they are capable of infiltrating most cancer tissues following activation by cytokines. In addition to their well-described role in inhibiting the early stage of tumor formation, NK cells are able to eradicate large solid tumors. Recent data confirm that NK cells are required for the induction of potent antitumor-specific CTL responses, by a mechanism involving DCs editing (Amakata et al., 2001; Walzer et al., 2005). Furthermore, NK cells can recognize tumors that might evade T-cell-mediated killing by aberrant human leukocyte antigen expression (Nielsen et al., 2012), indicating that NK cells actively participate in tumor immune surveillance.

The role of autophagy activation has been extended to the NK-mediated antitumor immune response. Recent evidence described that tumor cells can escape fully functional NK-mediated immune surveillance by activating autophagy under hypoxia (Baginska et al., 2013a; Viry et al., 2014a). Indeed, NK cells recognize and kill their targets by several

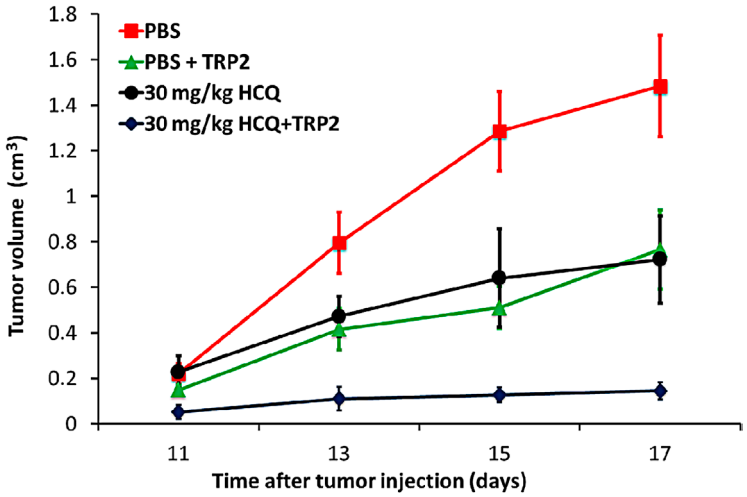

FIGURE 15.3 Autophagy inhibition by HCQ potentiates the antitumor effects of TRP-2(180–188) peptide vaccination. Growth curve of melanoma-engrafted tumor in the following group of C57BL/6 mice (n = 10): control unvaccinated mice treated with PBS; vaccinated mice treated with PBS (PBS + TRP2); unvaccinated mice treated with 30 mg/kg HCQ (30 mg/kg HCQ); and vaccinated mice treated with 30 mg/kg HCQ (30 mg/kg HCQ + TRP2). Data (mean ± SEM) are representative of three independent experiments. *Source: From Noman et al. (2011).*

mechanisms including the release of cytotoxic granules containing perforin and serine protease granzyme B (GZMB). It has been recently proposed that perforin and GZMB enter target cells by endocytosis and traffic to large endosomes named "gigantosomes" (Thiery et al., 2011). Subsequently, perforin is involved in the formation of pores in the membrane of the "gigantosome," leading to the gradual release of GZMB and the initiation of apoptotic cell death. The formation of amphisomes following the fusion between autophagic vacuoles and early endosomes appears to be necessary in some cases for the generation of autolysosomes. In this report (Baginska et al., 2013a), we described that the pro-apoptotic protein GZMB is selectively degraded upon autophagy activation in hypoxic cells, thereby inhibiting NK-mediated target cell apoptosis (Figure 15.4).

In light of the *in vitro* observations, we investigated whether targeting autophagy enhances *in vivo* NK-mediated antitumor immune response. BALB/c and C57BL/6 mice were transplanted with syngeneic murine 4T1 breast adenocarcinoma and B16-F10 melanoma tumor cells, respectively. We demonstrated that NK cells control *in vivo* B16-F10 and 4T1 tumor development, as the depletion of host NK cells significantly increases tumor growth. There is a significant decrease of tumor volume in autophagy-defective B16-F10 and 4T1 cells presumably as a consequence of potentiation of tumor cell killing by NK cells (Figure 15.5). Overall, this study underlines the inhibition of autophagy as a cutting-edge approach to overcome the suppressive effect of the hypoxic TME on the antitumor immune response.

More recently, the role of autophagy in regulating the NK-mediated immune response was extended to other tumor models. The clear-cell renal cell carcinoma is frequently

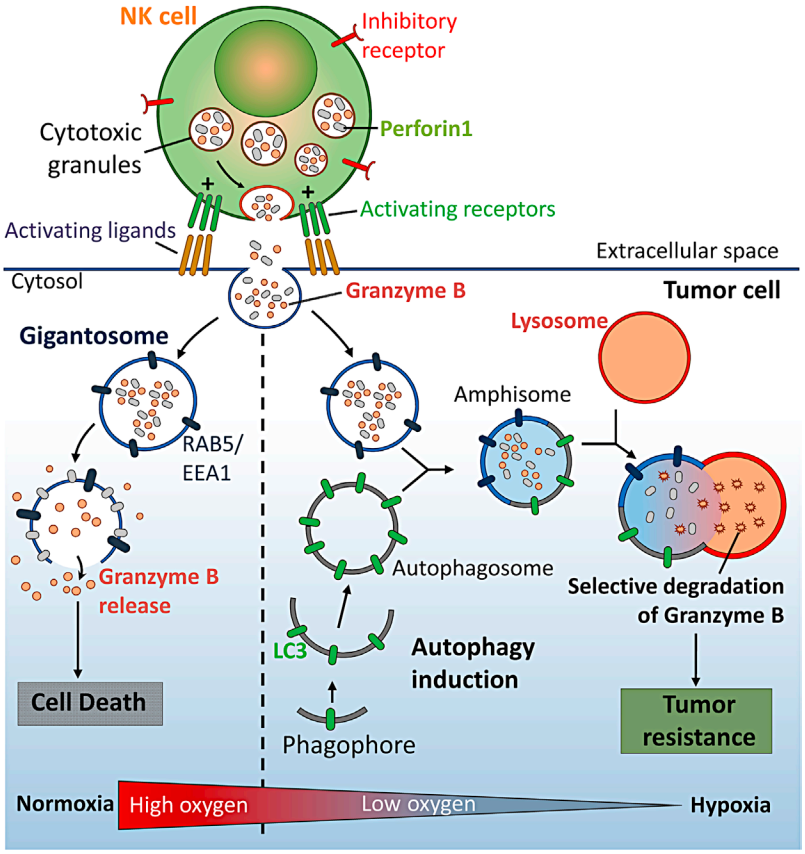

FIGURE 15.4 Hypoxia-induced autophagy in cancer cell acts as an intrinsic resistance mechanism to NK-mediated killing. Following recognition by NK cells, the cytolytic effectors perforin1 and granzyme B enter the target cells through endocytosis and traffic to enlarged endosomes called "gigantosomes." In normoxic cells, perforin1 forms pores in the "gigantosome" membrane, allowing granzyme B release and the initiation of cell death. In hypoxic cells, excessive autophagy leads to the fusion of "gigantosomes" with autophagosomes and the subsequent formation of amphisomes containing granzyme B and perforin1. The fusion of amphisome with lysosome triggers the selective degradation of granzyme B, making hypoxic tumor cell less sensitive to NK-mediated killing. *Source: From Viry et al. (2014b).*

associated with tumor-suppressor VHL gene mutations. Such mutations lead to the stabilization and accumulation of HIF-1α and HIF-2α and their target genes. Using VHL-mutated-786-O renal carcinoma cells, we reported that the subsequent stabilization of HIF-2α was strikingly associated with the resistance of 786-O cells to NK-mediated lysis. Targeting HIF-2α, or reconstitution of wild-type VHL in 786-O cells (hereafter referred to as WT-7 cells), significantly decreased the level of HIF-2α and restored the resistance of 786-O cells to NK-mediated lysis. These results highlight the critical role of HIF-2α in activating an intrinsic mechanism that makes renal cell carcinoma (RCC) less sensitive to NK cell attack. To gain further insight into the mechanism by which HIF-2α regulates RCC susceptibility to

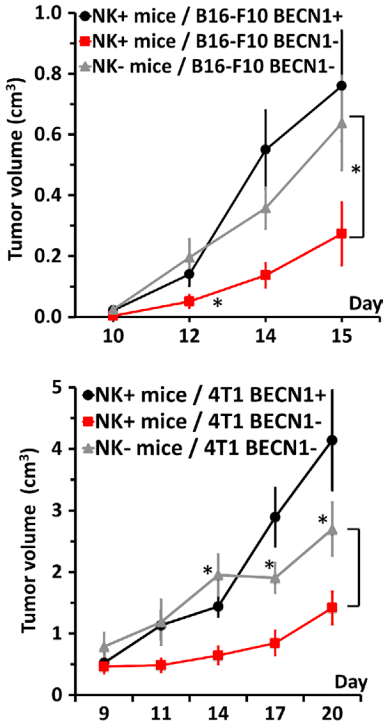

FIGURE 15.5 Targeting autophagy *in vivo* improves tumor elimination by NK cells. Autophagy-competent (BECN1+) or -defective (BECN1−) B16–F10 or 4T1 cells were injected subcutaneously (S.C) (Upper) or in the mammary fat pad (Lower), respectively, in control (NK+) and NK-depleted (NK−) C57BL/6 ($n = 7$) and BALB/c ($n = 10$) mice. Tumor growth was monitored using caliper measurements on the indicated days. Statistically significant differences are indicated by asterisks (*$P < 0.05$). *Source: From Baginska et al. (2013a).*

NK-mediated lysis, global gene expression profiling was performed on control and siRNA-HIF-2α-transfected 786-O cells. The result showed that the gene inositol 1,4,5-triphosphate receptor, type I (ITPR1) was overexpressed in 786-O as compared to HIF-2α-defective cells. Interestingly, targeting ITPR1 in 786-O was sufficient to dramatically restore NK-mediated lysis of these cells. These findings predict that the accumulation of HIF-2α in VHL-mutated 786-O cells leads to the overexpression of ITPR1 which subsequently alters the susceptibility to NK cell attack. Chromatin immunoprecipitation experiment further showed a HIF-2α enrichment of the ITPR1 promoter fragment containing HRE-7 in 786-O compared to WT-7 cells indicating that ITPR1 is a direct target of HIF-2α. Interestingly, in RCC patients, immunochemistry analysis showed a positive correlation between ITPR1 and HIF-2α expression. We next analyzed whether the accumulation of ITPR1 in 786-O cells was associated with the induction of autophagy. We were not able to detect any difference in the activation of autophagy in VHL-mutated 786-O and VHL-corrected WT-7 cells cultured without NK effectors. However, when cocultured with NK cells, only VHL-mutated 786-O cells were able to activate autophagy. This data strongly argue that the expression of ITPR1 is prerequisite for the induction of autophagy in RCC by a signal derived from NK cells. This was

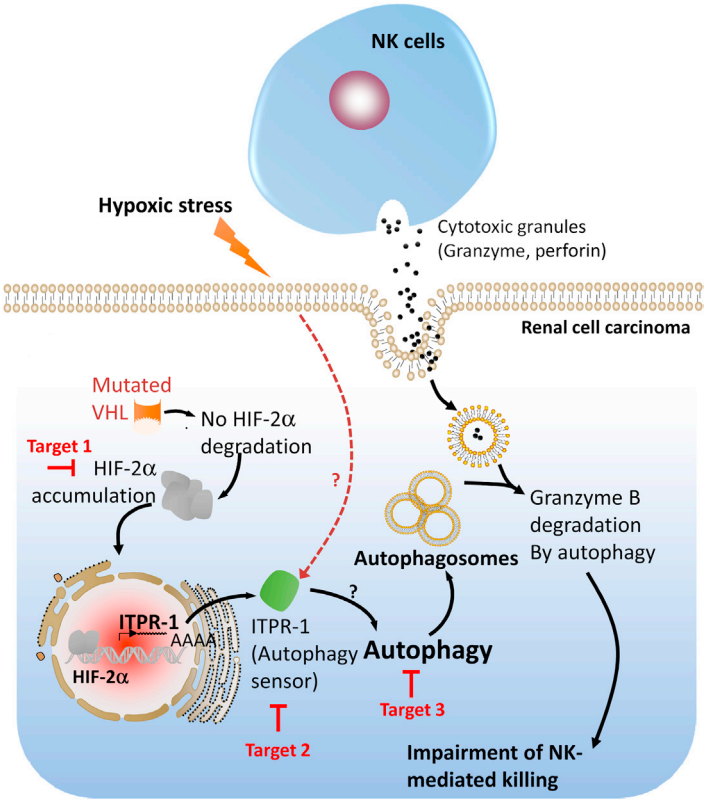

FIGURE 15.6 Targeting the autophagy sensor ITPR1 in renal cell carcinoma improves NK-mediated tumor cell killing. The expression of mutated VHL in renal cell carcinoma leads to the accumulation of HIF-2α. Accumulated HIF-2α translocates to the nucleus and induces the transcription of its target gene ITPR1. ITPR1 plays a key role in sensing a yet undefined signal derived from NK cells to activate autophagy by a mechanism that is not fully understood. The activation of autophagy in renal carcinoma cells leads to the degradation of NK-derived granzyme B and ultimately impairs NK-mediated tumor cell killing. At least three targets in this pathway (indicated in the figure as Targets 1, 2, and 3) may improve NK-mediated killing in renal cell carcinoma. *Source: Adapted from Messai et al. (2015).*

further supported by our data showing that targeting ITPR1 in 786-O cells abrogates the ability of NK cells to activate autophagy (Messai et al., 2014, 2015) (Figure 15.6).

As discussed above, the activation of autophagy in target tumor cells impairs NK-mediated tumor cell killing by degrading NK-derived GZMB. In accordance with this, higher level and activity of NK-derived GZMB were detected in WT-7 as compared to 786-O cells exhibiting increased level of autophagy. Targeting BECN1 in 786-O cells significantly restored GZMB level and activity. In light of these *in vitro* observations, the relevance of HIF-2α/ITPR1/autophagy pathway on NK-dependent antitumor immune response using Renca murine RCC was investigated. We demonstrated first that NK cells control *in vivo* Renca tumor development, by showing that the depletion of host NK cells significantly increased tumor growth. Furthermore, we observed a significant decrease of tumor volume

in mice engrafted with ITPR1-defective Renca cells as compared to control cells. This decrease might be due to the improvement of NK-mediated antitumor immune response. Consistent with this hypothesis, in NK-depleted mice, the regression of ITPR1 defective tumors was no longer observed. Taken together, these results suggest that inhibiting ITPR1/autophagy in tumors improves their elimination by NK cells *in vivo*. While several studies claim that autophagy inhibitors could improve anticancer therapies, other reports indicate that the use of autophagy inhibitors may also have negative effect in the context of cancer immunotherapy. This study highlights that targeting the autophagy sensor ITPR1 could be an alternative strategy to improve NK-mediated antitumor immune response in renal carcinoma (Messai et al., 2014, 2015).

Recently, the involvement of autophagy activation in the stability of the immune synapse between NK and hypoxic melanoma cells has emerged. Indeed, it has been shown that hypoxic stress increases the expression of one of the components of gap junctions, the connexin 43 (Cx43), in melanoma cells *via* HIF-1α transcriptional activity. Hypoxic melanoma cells displaying increased Cx43 expression were less susceptible to NK-mediated lysis compared to normoxic cells expressing moderate level of Cx43. Conversely, when overexpressed in normoxic tumor cells, Cx43 improves their susceptibility to NK-mediated killing. We showed that the IS between NK cells and normoxic melanoma cells is more stable and contains higher level of gap-junctional Cx43, whereas IS formed between NK cells and hypoxic cells is less stable and contains significant lower level of gap-junctional Cx43. It has been demonstrated that the activation of autophagy in hypoxic melanoma cells selectively degrades gap-junctional Cx43 leading to the destabilization of the IS and the impairment of NK-mediated killing. Inhibition of autophagy by genetic or pharmacological approaches, as well as expressing of undegradable form of Cx43, significantly restored its accumulation at the IS and improved NK-mediated lysis of hypoxic melanoma cells. This study provides evidence that hypoxic microenvironment negatively affects the immune surveillance of tumors by NK cells through the modulation of Cx43-mediated intercellular communications by autophagy.

Role of Autophagy in Tumor Cell Plasticity and Epithelial-to-Mesenchymal Transition

Emerging evidence has emphasized the multivariate and dynamic nature of the immune system and the ways in which tumor cells modulate themselves and their surroundings to escape immune surveillance. Indeed, phenotypic plasticity of tumor cells has serious implications in terms of immunological recognition and killing of the tumor (Holzel et al., 2013). Several mechanisms are associated with the acquisition of tumor cell plasticity and immune evasion, including loss of epithelial phenotype through epithelial-to-mesenchymal transition (EMT). In this section, we will discuss recent evidence revealing the involvement of autophagy in tumor cell plasticity and the emergence of immunoresistant variants.

EMT has become one of the most exciting fields in cancer biology. While its role in cancer cell invasion, metastasis, and drug resistance is well established (Nurwidya et al., 2012; Tsai and Yang, 2013), the molecular basis of EMT-induced immune escape remains unknown. EMT is a fundamental process in embryogenesis (Kalluri and Weinberg, 2009) that allows immobilized epithelial cells to migrate as single cells to localize in distant organs.

Mechanisms driving EMT in development have also been co-opted by carcinoma cells to promote cell plasticity, invasion, and metastasis (Chouaib et al., 2014). Most carcinoma cells exhibit a spectrum of EMT phenotypes or "epithelial cell plasticity," which is directly linked to histological grading and thus contributes to prognosis, stemness, immune suppression, and the development of resistant cell variants (Brabletz, 2012; Valastyan and Weinberg, 2011). Epithelial cells are characterized by a well-defined apico-basal polarity involved in the establishment of junctions between cells (Huang et al., 2012; Martin-Belmonte and Perez-Moreno, 2012). The adhesive receptor E-cadherin is a critical component of adherens junctions and it is often downregulated during tumor progression. Adherens junctions are thus most likely a major structure implicated in the control of epithelial cell plasticity (Thiery and Sleeman, 2006). Upon exposure to EMT inducers, polarized normal or transformed epithelial cells undergo morphological transition by launching a complex program of transcriptional, translational, and posttranslational mechanisms.

So far, the relationship between autophagy and EMT in tumors is not well elucidated and studies addressing this issue in the context of tumor immune response are emerging (Akalay et al., 2013a). However, the first evidence for the involvement of autophagy in EMT was described using epithelial breast adenocarcinoma MCF-7 cell line and its TNF-resistant clone (1001 cells) which undergo EMT (Janji et al., 2010) (Figure 15.7A). TNFα was previously described to induce autophagy in MCF-7 cells (Sivaprasad and Basu, 2008). Using autophagy dedicated microarray, we showed that the acquisition of mesenchymal phenotype in 1001 cells was correlated with significant modulation of 47 genes representing 20% of the genes represented on the autophagy microarray. Among this list, 34 genes were identified as significantly upregulated, while 13 were found significantly repressed in TNFα-resistant 1001 cells compared to TNFα-sensitive cells MCF-7. Thus, autophagy gene profiling experiments using autophagy microarray provided strong evidence that EMT is associated with a significant alteration of autophagy gene expression pattern suggesting a concomitant activation of autophagy in the cells. This hypothesis was supported by additional results showing the formation of numerous autophagosomes in 1001 compared to MCF-7 cells (Figure 15.7A). In addition, the expression of key autophagy genes was validated at mRNA and protein level. Data mining of autophagy microarray results performed by Ingenuity software highlights the involvement of MYC and HIF1-α downstream pathways in the activation of autophagy in TNF-resistant cells (Figure 15.7B).

Further study highlighting the link between autophagy and EMT reported that acquisition of EMT phenotype enables breast cancer cells to escape from T-cell-mediated lysis (Akalay et al., 2013b). Indeed, using the breast MCF-7-derived tumor cells that have undergone EMT following overexpression of wild-type SNAI1/SNAIL or the constitutively activated (SNAI1-6SA) protein, or by the acquisition of TNF/TNF-α resistance (2101 cells), we showed that EMT transcription factors are not the only way to induce an enhanced phenotypic plasticity resulting in breast cancer cell resistance to CTL. We also showed that the acquisition of resistance to TNF leads to the induction of EMT and the subsequent resistance to antigen-specific killer cells. It is worth noting that the acquisition of resistance to TNF and the high EMT score of TNF-resistant (2101) cells suggest the existence of a level of complexity in the EMT process in which multiple molecules act together to mediate EMT, rather than the master regulators acting on their own.

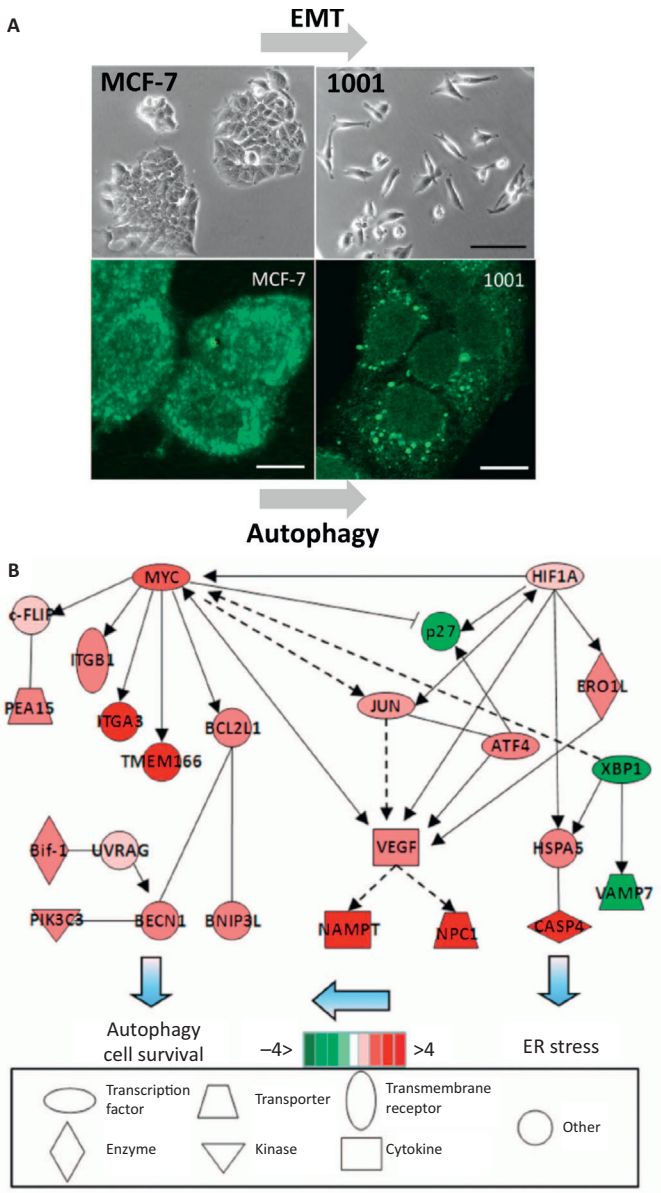

FIGURE 15.7 The involvement of autophagy in tumor cell plasticity and EMT. (A) The breast adenocarcinoma epithelial cells MCF-7 undergo EMT following the acquisition of resistance to TNF (clone 1001). Such EMT is associated with the induction of autophagy characterized by the formation of several autophagosomes (green dot–like structures) in 1001 cells. (B) Data mining of autophagy microarray results performed by Ingenuity software highlights the involvement of MYC and HIF1-α downstream pathways in the activation of autophagy in mesenchymal TNF-resistant cells. Solid lines indicate a direct interaction and dotted lines indicate an indirect interaction; arrows indicate that protein A acts directly (solid line) or indirectly (dotted line) on protein B. Green represents downregulation while red depicts upregulation in 1001 compared to MCF-7 cells. The intensity of color represents the average of log2 fold change from three independent experiments. Symbols affected to each gene reflect cell functions. *Source: From Moussay et al. (2011).*

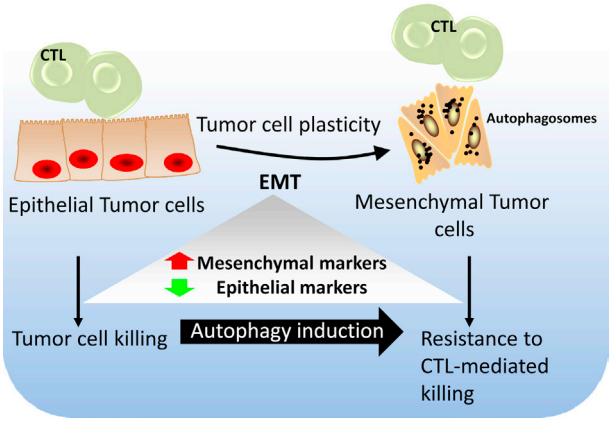

FIGURE 15.8 The acquisition of an EMT phenotype of tumor cells through loss of epithelial and gain of mesenchymal markers confers resistance to CTL-mediated lysis through autophagy induction.

Consistent with the role of autophagy as a cell-protective mechanism, this study further investigated whether the activation of the EMT program in tumor cells is associated with the induction of autophagy. The results showed that expression of SNAI1 in breast cancer cells induces an epithelial dedifferentiation program that coincides with a drastic change in cell morphology and the activation of autophagy flux (Figure 15.8). Interestingly, we found that BECN1 is upregulated in mesenchymal cells compared to epithelial cells.

Although the molecular mechanism by which the EMT program affects the expression of BECN1 remains to be addressed, several lines of evidence indicate that this may be related to SNAI1- or EMT-dependent repression of microRNAs involved in modulation of BECN1 expression. Indeed, it has been reported that MIR30A inhibits the expression of BECN1, and that the transcription factors SNAI1 and ZEB1 bind to E-boxes in the MIR34A/B/C promoters, thereby repressing MIR34A and MIR34B/C expression. While much remains to be learned mechanistically, this result extended the role of SNAI1 as a regulator of autophagy and paved the way to an interesting topic of research. Although targeting BECN1 in mesenchymal cells is sufficient to restore CTL-mediated tumor cell lysis, it has no effect on cell morphology and the expression of EMT markers. This finding suggests that autophagy is a downstream target of the EMT program in breast cancer cells (Akalay et al., 2013a,b).

AUTOPHAGY INHIBITION IN CANCER THERAPY: FRIEND OR FOE?

The initial rationale for using autophagy inhibition in anticancer therapy was generated by research revealing that autophagy is upregulated in response to external stresses allowing resistance of tumor cells to chemotherapy (Sui et al., 2013), radiotherapy (Chaachouay et al., 2011), and immunotherapy (Baginska et al., 2013a,b; Noman et al., 2011, 2012). Combining autophagy inhibition with chemotherapy agents may therefore increase their therapeutic efficacy (Mancias and Kimmelman, 2011). On the basis of these observations, it

is not surprising that autophagy has emerged as a potential therapeutic target, and research efforts have intensified to develop autophagy inhibitors that could be used in combination with anticancer therapies.

Pharmacological inhibitors of autophagy identified so far can be classified in two main groups depending on which stage of the autophagy process is targeted. Sequestration inhibitors, such as 3-methyladenine (3-MA), LY294002, and wortmannin, act at early stage of the autophagy pathway by inhibiting the class III phosphatidylinositol-3 kinase (PI3K). Recently, a potent small molecule inhibitor of autophagy, called spautin-1, was identified, which causes the degradation of the class III PI3K complex by targeting BECN1 (Liu et al., 2011). Most of other inhibitory compounds act at later stages. Microtubule poisons, such as vinca alkaloids, taxanes, nocodazole, and colchicine, cause blockade of autophagosome and lysosome fusion. Inhibitors of lysosomal enzymes (e.g., leupeptin, pepstatin A, and E-64d) or compounds that elevate lysosomal pH (e.g., bafilomycin A1, chloroquine (CQ)) impair autophagy through the inhibition of cargo degradation by lysosomal hydrolases (Klionsky et al., 2012). CQ and its derivative HCQ have long been used as antimalarial and antirheumatic drug, and they were the only autophagy inhibitors approved by the US Food and Drug Administration. As single agent, CQ has shown anticancer activity in lymphoma (Maclean et al., 2008), pancreatic (Yang et al., 2011), and breast cancers (Jiang et al., 2010). In addition, the use of CQ or HCQ in combination with conventional therapies has provided convincing results in preclinical models (Amaravadi et al., 2011). Indeed, autophagy blockade enhances anticancer effects of apoptosis-inducing agents (Amaravadi et al., 2007) and Src family kinase inhibitors (Wu et al., 2010). Moreover, this strategy has shown promising results on patients' survival in the first phase III clinical trial using CQ as adjuvant treatment to conventional anticancer therapy for glioblastoma (Briceno et al., 2007). Currently, more than 30 clinical trials are registered with the National Cancer Institute to evaluate the effects of autophagy inhibition in a variety of human cancers (http://clinicaltrials.gov). Table 15.1 summarizes the clinical trials involving CQ or HCQ in combinational treatment of refractory malignancies.

Encouraging preclinical results support the use of autophagy inhibitors to induce a potent antitumor immune response. We recently demonstrated that increased autophagy in tumor cell suppressed the antitumor immune response, and that autophagy blockade enhanced CTL- and NK-mediated tumor cell killing once they have been activated to lyse tumor cells (Baginska et al., 2013a; Noman et al., 2011). These data imply that strategies based on adoptive transfer of T cells, DCs vaccines, or administration of antibodies or recombinant cytokines such as interleukine-2 (IL-2), could be more effective if the blockade of autophagy is effective in tumor cells (Amaravadi et al., 2011). Indeed, Liang et al. (2012) showed that the combination of high dose of IL-2 with CQ promotes long-term survival, decreased toxicity, and enhanced immune cell proliferation and infiltration in advanced murine metastatic liver tumor model (Liang et al., 2012). In keeping with this, a clinical protocol has been initiated to evaluate the combinational administration of IL-2 and HCQ in patients with advanced renal cell cancer.

While experimental and preclinical data are generally supportive of incorporating anti-autophagy therapies in cancer treatment trials, it has been observed in some circumstances that inhibition of autophagy decreases therapeutic efficacy and therefore acting

as double-edged sword. It is now clearly established that autophagy plays also a crucial role in increasing the immunogenicity of the tumor cell, and actively participates in the antigen processing and the subsequent activation of the effectors T cells. Thus, induction of autophagy may be exploited as adjuvant strategy to stimulate the antitumor immune response. Indeed, cancer cell–associated autophagy contributes to immunogenic cell death (ICD) through the release/exposure of immunostimulatory danger signals that stimulate the antitumor immune response. Such signaling molecules include secreted ATP, surface-exposed calreticulin, and high-mobility group box 1 release (Maes et al., 2013; Viry et al., 2014b). This important role of autophagy in eliciting ICD was reported in a recent study showing how autophagy-competent cells, but not autophagy-deficient cells, enable to release ATP and recruit DCs and T lymphocytes into the tumor bed in mice (Ma et al., 2013). Recently, the same group has confirmed that chemotherapy-induced autophagy in cancer cells determines the outcome of melanoma therapy. Systemic treatment with the anthracycline Mitoxantrone reduced the growth of autophagy-competent melanomas, but not autophagy-deficient tumors. This growth-inhibitory activity of Mitoxantone, observed on autophagy-competent melanomas, was shown to be mediated through CD4+ and CD8+ T lymphocytes, suggesting that autophagy is required to trigger a potent anticancer response (Michaud et al., 2014).

Tumor-derived autophagosomes vaccine, or autophagosomes loaded onto DCs, in combination with agents that reverse the immune suppression (e.g., IFN-γ, TLR agonist) enhanced the antitumor response against 3LL Lewis lung carcinoma and B16-F10 melanoma (Li et al., 2011). A phase I/II is currently ongoing to investigate the efficacy of such vaccine in patients with non–small-cell lung carcinoma. Furthermore, it has been described that autophagosomes are essential carriers for cross-presentation of tumor-associated antigens (Li et al., 2008). They have demonstrated that induction of autophagy in tumor cells, following exposure to alpha-tocopheryloxyacetic acid (alpha-TEA), generates double membrane–bound autophagosomes containing antigens that enhance the cross-priming of CD8+ T lymphocytes. Moreover, inhibition of autophagy, with 3-MA or by specific silencing of *ATG12*, partially blocks T-cell activation. The authors showed that vaccination with DCs pulsed with autophagosome-enriched fraction, derived from tumor cells treated *in vitro* with alpha-TEA, decreased lung metastasis and increased survival of tumor-bearing mice (Li et al., 2012). Therefore, an autophagy inducer, such alpha-TEA, might be exploited as adjuvant therapy to improve efficacy of immune modulator of T-cell response (e.g., anti-CTLA-4 antibody). Recently, Amaravadi et al. (2011) highlighted that cancer patients are suffering from a "systemic autophagic syndrome," meaning that autophagy is activated in tumor cells while suppressed in immune effectors (Amaravadi et al., 2011). Taken together, these observations emphasize that future therapeutic approaches may combine *ex vivo* autophagy induction in immune cells and systemic autophagy inhibition to improve efficacy of immunotherapies.

Based on studies described above, it is important to highlight that therapeutic strategies targeting autophagy in tumor cells must consider the potential negative impact on antitumor immunity. The key question that emerges is the net outcome of the autophagy inhibitor in clinic. Furthermore, understanding to which extent and under which circumstances inducers and/or inhibitors of autophagy affect the therapeutic efficacy of anticancer treatments will be of great importance to improve the rational use of such modulators.

TABLE 15.1 Examples of Clinical Trials Involving Autophagy Inhibitors in Combination with Anticancer Therapies (http://cancer.gov/clinicaltrials)

Cancer Type	Drug Intervention	Phase Status	Clinical Trial ID	Title of the Clinical Trial
Pancreatic cancer	HCQ +Gemcitabine +Abraxane	I/II Active	NCT01506973	A Phase I/II/Pharmacodynamic Study of HCQ in Combination With Gemcitabine/Abraxane to Inhibit Autophagy in Pancreatic Cancer
Breast cancer	HCQ	II Active	NCT01292408	Autophagy Inhibition Using HCQ in Breast Cancer Patients
NSCL cancer	HCQ +Paclitaxel +Carboplatin +Bevacizumab	II Active	NCT01649947	Modulation of Autophagy in Patients With Advanced/Recurrent NSCL Cancer - Phase II
Renal cancer	HCQ +RAD001	I/II Active	NCT01510119	Autophagy Inhibition to Augment mTOR Inhibition: A Phase I/II Trial of RAD001 and HCQ in Patients With Previously Treated Renal Cell Carcinoma
SCLC	CQ +Chemotherapy +Radiotherapy	I Active	NCT00969306	CQ as an Anti-Autophagy Drug in Stage IV SCLC Patients
Colorectal cancer	HCQ +FOLFOX +Bevacizumab	I/II Active	NCT01206530	FOLFOX/Bevacizumab/HCQ in Colorectal Cancer
Solid tumors	HCQ +Sorafenib	I Active	NCT01634893	Oral HCQ Plus Oral Sorafenib to Treat Patients With Refractory or Relapsed Solid Tumors
Solid tumors	CQ +Carboplatin +Gemcitabine	I Active	NCT02071537	CQ in Combination With Carboplatin/Gemcitabine in Advanced Solid Tumors
Solid tumors	HCQ +Temsirolimus	I Active	NCT00909831	HCQ and Temsirolimus in Treating Patients With Metastatic Solid Tumors That Have Not Responded to Treatment
Chronic myeloid leukemia	HCQ +Imatinib mesylate	II Active	NCT01227135	Imatinib Mesylate With or Without HCQ in Treating Patients With Chronic Myeloid Leukemia
Pancreatic cancer	HCQ +Gemcitabine +Nab-Paclitaxel	II Active	NCT01978184	Randomized Phase II Trial of Pre-Operative Gemcitabine and Nab Paclitacel With or Without HCQ
Melanoma, prostate or kidney cancers	HCQ +MK2206	I Active	NCT01480154	Akt Inhibitor MK2206 and HCQ in Treating Patients With Advanced Solid Tumors, Melanoma, Prostate or Kidney Cancer

(Continued)

TABLE 15.1 (Continued)

Cancer Type	Drug Intervention	Phase Status	Clinical Trial ID	Title of the Clinical Trial
Multiple myeloma	HCQ +Cyclophosphamide +Dexamethasone +Sirolimus	I Active	NCT01689987	HCQ, Cyclophosphamide, Dexamethasone, and Sirolimus in Treating Patients With Relapsed or Refractory Multiple Myeloma
Solid tumors	HCQ +Vorinostat	I Active	NCT01023737	HCQ Plus Vorinostat in Advanced Solid Tumors
Pancreatic cancer	HCQ +Radiotherapy	II Active	NCT01494155	Short Course Radiation Therapy With Proton or Photon Beam Capecitabine and HCQ for Resectable Pancreatic Cancer
Glioma	HCQ +Radiotherapy	II Active	NCT01602588	A Randomised Trial Investigating the Additional Benefit of HCQ to Short Course Radiotherapy (SCRT) in Patients Aged 70 Years and Older With High Grade Gliomas
Soft tissue sarcoma	HCQ +Sirolimus	II Active	NCT01842594	A Phase II Trial of Combined HCQ and Sirolimus in Soft Tissue Sarcoma
Melanoma	HCQ +Vemurafenib	I Active	NCT01897116	A Phase I Trial of Vemurafenib and HCQ in Patients With Advanced BRAF Mutant Melanoma
Renal cancer	HCQ +Aldesleukin	I/II Active	NCT01550367	Study of HCQ and Aldesleukin in Renal Cell Carcinoma Patients
Colorectal cancer	HCQ +Vorinostat	II Approved	NCT02316340	Vorinostat Plus HCQ Versus Regorafenib in Colorectal Cancer
Advanced cancer	HCQ +Vorinostat or Sirolimus	I Active	NCT01266057	Sirolimus or Vorinostat and HCQ in Advanced Cancer
Prostate cancer	HCQ +Navitoclax +Abiraterone acetate	II Active	NCT01828476	Navitoclax and Abiraterone Acetate With or Without HCQ in Treating Patients With Progressive Metastatic Castrate Refractory Prostate Cancer
Melanoma	HCQ +Dabrafenib +Trametinib	I/II Active	NCT02257424	The BAMM Trial: BRAF, Autophagy and MEK Inhibition in Metastatic Melanoma: A Phase I/II Trial of Dabrafenib, Trametinib and HCQ in Patients With Advanced BRAF Mutant Melanoma
Solid tumors	HCQ +Temozolomide	I Active	NCT00714181	HCQ and Temozolomide in Treating Patients With Metastatic or Unresectable Solid Tumors

(Continued)

TABLE 15.1 (Continued)

Cancer Type	Drug Intervention	Phase Status	Clinical Trial ID	Title of the Clinical Trial
Multiple myeloma	HCQ +Bortezomib	I/II Active	NCT00568880	HCQ and Bortezomib in Treating Patients With Relapsed or Refractory Multiple Myeloma
Pancreatic cancer	HCQ +Gemcitabine	I/II Closed	NCT01128296	Study of Pre-surgery Gemcitabine Plus HCQ in Stage IIb or III Adenocarcinoma of the Pancreas
Colorectal cancer	HCQ +Capecitabine +Oxaliplatin +Bevacizumab	II Closed		HCQ, Capecitabine, Oxaliplatin, and Bevacizumab in Treating Patients With Metastatic Colorectal Cancer
Solid tumors	HCQ +Sunitinib malate	I Closed	NCT00813423	Sunitinib Malate and HCQ in Treating Patients With Advanced Solid Tumors That Have Not Responded to Chemotherapy
Multiple myeloma	Rapamycin or HCQ +Cyclophosphamide +Dexamethasone	NS Closed	NCT01396200	Cyclophosphamide and Pulse Dexamethasone With Rapamycin or HCQ
Glioblastoma	HCQ +Radiotherapy +Temozolomide	I/II Closed	NCT00486603	HCQ, Radiation Therapy, and Temozolomide in Treating Patients With Newly Diagnosed Glioblastoma Multiforme

CQ, chloroquine; HCQ, hydroxychloroquine; NSCL, non–small-cell lung; SCLC, small-cell lung cancer; NS, not specified.

CONCLUDING REMARKS

Although the multiple roles of autophagy in cancer require further investigation, it is obvious that autophagy is directly involved in several important physiological and pathological processes including cancer cell death, response to stress, metabolic adaptation, and resistance to anticancer therapies. Considering the potential seminal role of autophagy in both normal and abnormal cells, it is difficult to draw a clear conclusion whether autophagy has to be stimulated or repressed. Accordingly, the role of autophagy in cancer raises a number of intriguing questions. Does autophagy play a direct or indirect role in cancer development and progression? If it does, what is its exact contribution? Can autophagy be exploited as a mean of enhancing cancer therapies? From the data discussed in this chapter it appears that, at least in the context of cancer immunotherapy, both strategies are promising. However, harnessing autophagy for therapeutic purposes will require careful consideration on whether, when, and how autophagy is induced as a pro-survival mechanism, or is recruited to promote cancer cell killing. To date, most of the studies have focused on the impact of autophagy on tumor cells themselves, but it should be more accurate to consider

autophagy in the context of the TME. It has been increasingly clear that, in the context of tumor immunity, autophagy may influence the cross talk between cancer and immune cells, leading either to immunoevasion or immunostimulation. Further knowledge on the impact of autophagy in tumor cells as well as in the TME is necessary to tailor therapies that selectively block suppressive mechanisms that impede antitumor response while promoting the antitumor immunity.

Furthermore, before having autophagy modulators to be incorporated into clinical settings, several questions require to be addressed. How should autophagy modulation be accomplished? Which tumors will be sensitive to autophagy modulation, or which patients will benefit from autophagy modulation? To address these questions an extensive tumor characterization is required, considering the following. Different tumor types show various responses to autophagy modulation and may differ in sensitivity. The composition of the TME and the hypoxic characteristic of the tumor could influence treatment efficacy. Basal levels of autophagy in tumors can also affect response. Nevertheless, there is evidence that certain tumor types, mainly those harboring oncogenic RAS mutation, will benefit from autophagy inhibition.

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References

Akalay, I., Janji, B., Hasmim, M., et al., 2013a. Epithelial-to-mesenchymal transition and autophagy induction in breast carcinoma promote escape from T-cell-mediated lysis. Cancer Res. 73, 2418–2427.

Akalay, I., Janji, B., Hasmim, M., et al., 2013b. EMT impairs breast carcinoma cell susceptibility to CTL-mediated lysis through autophagy induction. Autophagy 9, 1104–1106.

Amakata, Y., Fujiyama, Y., Andoh, A., et al., 2001. Mechanism of NK cell activation induced by coculture with dendritic cells derived from peripheral blood monocytes. Clin. Exp. Immunol. 124, 214–222.

Amaravadi, R.K., Yu, D., Lum, J.J., et al., 2007. Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. J. Clin. Invest. 117, 326–336.

Amaravadi, R.K., Lippincott-Schwartz, J., Yin, X.M., et al., 2011. Principles and current strategies for targeting autophagy for cancer treatment. Clin. Cancer Res. 17, 654–666.

Baginska, J., Viry, E., Berchem, G., et al., 2013a. Granzyme B degradation by autophagy decreases tumor cell susceptibility to natural killer-mediated lysis under hypoxia. Proc. Natl. Acad. Sci. U.S.A. 110, 17450–17455.

Baginska, J., Viry, E., Paggetti, J., et al., 2013b. The critical role of the tumor microenvironment in shaping natural killer cell-mediated anti-tumor immunity. Front. Immunol. 4, 490.

Balkwill, F.R., Capasso, M., Hagemann, T., 2012. The tumor microenvironment at a glance. J. Cell Sci. 125, 5591–5596.

Bellot, G., Garcia-Medina, R., Gounon, P., et al., 2009. Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains. Mol. Cell Biol. 29, 2570–2581.

Brabletz, T., 2012. EMT and MET in metastasis: where are the cancer stem cells? Cancer Cell 22, 699–701.

Briceno, E., Calderon, A., Sotelo, J., 2007. Institutional experience with chloroquine as an adjuvant to the therapy for glioblastoma multiforme. Surg. Neurol. 67, 388–391.

Buchser, W.J., Laskow, T.C., Pavlik, P.J., et al., 2012. Cell-mediated autophagy promotes cancer cell survival. Cancer Res. 72, 2970–2979.
Chaachouay, H., Ohneseit, P., Toulany, M., et al., 2011. Autophagy contributes to resistance of tumor cells to ionizing radiation. Radiother. Oncol. 99, 287–292.
Chouaib, S., Janji, B., Tittarelli, A., et al., 2014. Tumor plasticity interferes with anti-tumor immunity. Crit. Rev. Immunol. 34, 91–102.
Cullen, S.P., Brunet, M., Martin, S.J., 2010. Granzymes in cancer and immunity. Cell Death Differ. 17, 616–623.
Dustin, M.L., Cooper, J.A., 2000. The immunological synapse and the actin cytoskeleton: molecular hardware for T cell signaling. Nat. Immunol. 1, 23–29.
Fridman, W.H., Pages, F., Sautes-Fridman, C., et al., 2012. The immune contexture in human tumours: impact on clinical outcome. Nat. Rev. Cancer 12, 298–306.
Grakoui, A., Bromley, S.K., Sumen, C., et al., 1999. The immunological synapse: a molecular machine controlling T cell activation. Science 285, 221–227.
Hamai, A., Benlalam, H., Meslin, F., et al., 2010. Immune surveillance of human cancer: if the cytotoxic T-lymphocytes play the music, does the tumoral system call the tune? Tissue Antigens 75, 1–8.
Harris, A.L., 2002. Hypoxia—a key regulatory factor in tumour growth. Nat. Rev. Cancer 2, 38–47.
Holzel, M., Bovier, A., Tuting, T., 2013. Plasticity of tumour and immune cells: a source of heterogeneity and a cause for therapy resistance? Nat. Rev. Cancer 13, 365–376.
Huang, R.Y., Guilford, P., Thiery, J.P., 2012. Early events in cell adhesion and polarity during epithelial-mesenchymal transition. J. Cell Sci. 125, 4417–4422.
Janji, B., Vallar, L., Al Tanoury, Z., et al., 2010. The actin filament cross-linker L-plastin confers resistance to TNF-alpha in MCF-7 breast cancer cells in a phosphorylation-dependent manner. J. Cell. Mol. Med. 14, 1264–1275.
Jiang, P.D., Zhao, Y.L., Deng, X.Q., et al., 2010. Antitumor and antimetastatic activities of chloroquine diphosphate in a murine model of breast cancer. Biomed. Pharmacother. 64, 609–614.
Kalluri, R., Weinberg, R.A., 2009. The basics of epithelial-mesenchymal transition. J. Clin. Invest. 119, 1420–1428.
Ke, Q., Costa, M., 2006. Hypoxia-inducible factor-1 (HIF-1). Mol. Pharmacol. 70, 1469–1480.
Klionsky, D.J., Abdalla, F.C., Abeliovich, H., et al., 2012. Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy 8, 445–544.
Kothari, S., Cizeau, J., McMillan-Ward, E., et al., 2003. BNIP3 plays a role in hypoxic cell death in human epithelial cells that is inhibited by growth factors EGF and IGF. Oncogene 22, 4734–4744.
Li, Y., Wang, L.X., Yang, G., et al., 2008. Efficient cross-presentation depends on autophagy in tumor cells. Cancer Res. 68, 6889–6895.
Li, Y., Wang, L.X., Pang, P., et al., 2011. Tumor-derived autophagosome vaccine: mechanism of cross-presentation and therapeutic efficacy. Clin. Cancer Res. 17, 7047–7057.
Li, Y., Hahn, T., Garrison, K., et al., 2012. The vitamin E analogue alpha-TEA stimulates tumor autophagy and enhances antigen cross-presentation. Cancer Res. 72, 3535–3545.
Liang, X., De Vera, M.E., Buchser, W.J., et al., 2012. Inhibiting systemic autophagy during interleukin 2 immunotherapy promotes long-term tumor regression. Cancer Res. 72, 2791–2801.
Liu, J., Xia, H., Kim, M., et al., 2011. Beclin1 controls the levels of p53 by regulating the deubiquitination activity of USP10 and USP13. Cell 147, 223–234.
Lotze, M.T., Buchser, W.J., Liang, X., 2012. Blocking the interleukin 2 (IL2)-induced systemic autophagic syndrome promotes profound antitumor effects and limits toxicity. Autophagy 8, 1264–1266.
Lum, J.J., Bauer, D.E., Kong, M., et al., 2005. Growth factor regulation of autophagy and cell survival in the absence of apoptosis. Cell 120, 237–248.
Ma, Y., Galluzzi, L., Zitvogel, L., et al., 2013. Autophagy and cellular immune responses. Immunity 39, 211–227.
Maclean, K.H., Dorsey, F.C., Cleveland, J.L., et al., 2008. Targeting lysosomal degradation induces p53-dependent cell death and prevents cancer in mouse models of lymphomagenesis. J. Clin. Invest. 118, 79–88.
Maes, H., Rubio, N., Garg, A.D., et al., 2013. Autophagy: shaping the tumor microenvironment and therapeutic response. Trends Mol. Med. 19, 428–446.
Majmundar, A.J., Wong, W.J., Simon, M.C., 2010. Hypoxia-inducible factors and the response to hypoxic stress. Mol. Cell 40, 294–309.
Mancias, J.D., Kimmelman, A.C., 2011. Targeting autophagy addiction in cancer. Oncotarget 2, 1302–1306.

Martin-Belmonte, F., Perez-Moreno, M., 2012. Epithelial cell polarity, stem cells and cancer. Nat. Rev. Cancer 12, 23–38.
Mazure, N.M., Pouyssegur, J., 2009. Atypical BH3-domains of BNIP3 and BNIP3L lead to autophagy in hypoxia. Autophagy 5, 868–869.
Mazure, N.M., Pouyssegur, J., 2010. Hypoxia-induced autophagy: cell death or cell survival? Curr. Opin. Cell Biol. 22, 177–180.
Messai, Y., Noman, M.Z., Hasmim, M., et al., 2014. ITPR1 protects renal cancer cells against natural killer cells by inducing autophagy. Cancer Res. 74, 6820–6832.
Messai, Y., Noman, M.Z., Janji, B., et al., 2015 Feb 25. The autophagy sensor ITPR1 protects renal carcinoma cells from NK-mediated killing. Autophagy 0. [Epub ahead of print].
Michaud, M., Martins, I., Sukkurwala, A.Q., et al., 2011. Autophagy-dependent anticancer immune responses induced by chemotherapeutic agents in mice. Science 334, 1573–1577.
Michaud, M., Xie, X., Bravo-San Pedro, J.M., et al., 2014. An autophagy-dependent anticancer immune response determines the efficacy of melanoma chemotherapy. Oncoimmunology 3, e944047.
Moussay, E., Kaoma, T., Baginska, J., et al., 2011. The acquisition of resistance to TNFalpha in breast cancer cells is associated with constitutive activation of autophagy as revealed by a transcriptome analysis using a custom microarray. Autophagy 7, 760–770.
Nielsen, N., Odum, N., Urso, B., et al., 2012. Cytotoxicity of CD56(bright) NK cells towards autologous activated CD4+ T cells is mediated through NKG2D, LFA-1 and TRAIL and dampened via CD94/NKG2A. PLoS One 7, e31959.
Noman, M.Z., Janji, B., Kaminska, B., et al., 2011. Blocking hypoxia-induced autophagy in tumors restores cytotoxic T-cell activity and promotes regression. Cancer Res. 71, 5976–5986.
Noman, M.Z., Janji, B., Berchem, G., et al., 2012. Hypoxia-induced autophagy: a new player in cancer immunotherapy? Autophagy 8, 704–706.
Nurwidya, F., Takahashi, F., Murakami, A., et al., 2012. Epithelial mesenchymal transition in drug resistance and metastasis of lung cancer. Cancer Res. Treat. 44, 151–156.
Papandreou, I., Lim, A.L., Laderoute, K., et al., 2008. Hypoxia signals autophagy in tumor cells via AMPK activity, independent of HIF-1, BNIP3, and BNIP3L. Cell Death Differ. 15, 1572–1581.
Rouschop, K.M., van den Beucken, T., Dubois, L., et al., 2010. The unfolded protein response protects human tumor cells during hypoxia through regulation of the autophagy genes MAP1LC3B and ATG5. J. Clin. Invest. 120, 127–141.
Shresta, S., MacIvor, D.M., Heusel, J.W., et al., 1995. Natural killer and lymphokine-activated killer cells require granzyme B for the rapid induction of apoptosis in susceptible target cells. Proc. Natl. Acad. Sci. U.S.A. 92, 5679–5683.
Sivaprasad, U., Basu, A., 2008. Inhibition of ERK attenuates autophagy and potentiates tumour necrosis factor-alpha-induced cell death in MCF-7 cells. J. Cell. Mol. Med. 12, 1265–1271.
Smyth, M.J., Godfrey, D.I., Trapani, J.A., 2001. A fresh look at tumor immunosurveillance and immunotherapy. Nat. Immunol. 2, 293–299.
Sui, X., Chen, R., Wang, Z., et al., 2013. Autophagy and chemotherapy resistance: a promising therapeutic target for cancer treatment. Cell Death Dis. 4, e838.
Tachibana, T., Onodera, H., Tsuruyama, T., et al., 2005. Increased intratumor Valpha24-positive natural killer T cells: a prognostic factor for primary colorectal carcinomas. Clin. Cancer Res. 11, 7322–7327.
Thiery, J.P., Sleeman, J.P., 2006. Complex networks orchestrate epithelial-mesenchymal transitions. Nat. Rev. Mol. Cell Biol. 7, 131–142.
Thiery, J., Keefe, D., Boulant, S., et al., 2011. Perforin pores in the endosomal membrane trigger the release of endocytosed granzyme B into the cytosol of target cells. Nat. Immunol. 12, 770–777.
Tsai, J.H., Yang, J., 2013. Epithelial-mesenchymal plasticity in carcinoma metastasis. Genes Dev. 27, 2192–2206.
Valastyan, S., Weinberg, R.A., 2011. Tumor metastasis: molecular insights and evolving paradigms. Cell 147, 275–292.
Viry, E., Baginska, J., Berchem, G., et al., 2014a. Autophagic degradation of GZMB/granzyme B: a new mechanism of hypoxic tumor cell escape from natural killer cell-mediated lysis. Autophagy 10, 173–175.
Viry, E., Paggetti, J., Baginska, J., et al., 2014b. Autophagy: an adaptive metabolic response to stress shaping the antitumor immunity. Biochem. Pharmacol. 92, 31–42.

Walzer, T., Dalod, M., Vivier, E., et al., 2005. Natural killer cell-dendritic cell crosstalk in the initiation of immune responses. Expert Opin. Biol. Ther. 5 (Suppl. 1), S49–S59.
Watzl, C., Long, E.O., 2000. Exposing tumor cells to killer cell attack. Nat. Med. 6, 867–868.
White, E., 2012. Deconvoluting the context-dependent role for autophagy in cancer. Nat. Rev. Cancer 12, 401–410.
Whiteside, T.L., 2008. The tumor microenvironment and its role in promoting tumor growth. Oncogene 27, 5904–5912.
Wildenberg, M.E., Vos, A.C., Wolfkamp, S.C., et al., 2012. Autophagy attenuates the adaptive immune response by destabilizing the immunologic synapse. Gastroenterology 142, 1493–1503. e1496.
Wu, Z., Chang, P.C., Yang, J.C., et al., 2010. Autophagy blockade sensitizes prostate cancer cells towards Src family kinase inhibitors. Genes Cancer 1, 40–49.
Yang, S., Wang, X., Contino, G., et al., 2011. Pancreatic cancers require autophagy for tumor growth. Genes Dev. 25, 717–729.

CHAPTER

16

Omega-3 DHA and EPA Conjugates Trigger Autophagy Through PPARγ Activation in Human Breast Cancer Cells

Daniela Bonofiglio, Marilena Lanzino, Cinzia Giordano, Stefania Catalano and Sebastiano Andò

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Abstract

The omega-3 docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) may form conjugates with amines that have potential health benefits against common diseases including cancers. We demonstrated that conjugates of both DHA and EPA with ethanolamine (DHEA and EPEA) or dopamine (DHADA and EPADA), synthesized in our laboratory, were able to significantly reduce cell viability in different breast cancer cell lines while they did not elicit any effects in nontumorigenic breast epithelial cells. In line with previous findings reporting that DHA and EPA are ligands of peroxisome proliferator-activated receptor (PPAR)γ, we also showed that PPARγ represents a mediator of DHA/EPA conjugates biological effects. In

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breast cancer cells, these compounds enhanced PPARγ expression and stimulated the PPAR response element dependent transcription, as confirmed by the increased expression of PPARγ target gene *PTEN* (*phosphatase and tensin homolog on chromosome ten*), responsible for the inhibition of AKT–mTOR (mammalian target of rapamycin) pathways. Both ethanolamine- or dopamine conjugates triggered autophagy in breast cancer cells through upregulation of the autophagy-related protein, Beclin-1. Indeed, DHEA and EPEA treatment by inducing phosphorylation of Bcl-2 promoted its dissociation from Beclin-1 which resulted in increased unbound levels of Beclin-1. Instead, DHADA and EPADA exposure enhanced Beclin-1 levels by inducing its gene promoter transcriptional activity through recruitment of PPARγ at the retinoic-X-receptor binding region within the Beclin-1 promoter. Moreover, all conjugates enhanced autophagosome formation, evaluated by microtubule-associated protein 1 light chain 3 and monodansylcadaverine labeling, that was prevented by knocking down PPARγ expression or using the PPARγ antagonist GW9662, confirming the direct involvement of PPARγ in these effects. Noteworthy, DHADA- and EPADA-induced autophagy was followed by apoptotic cell death as evidenced by PARP (poly (ADP-ribose) polymerase) cleavage and DNA laddering. On the basis of the evidence that omega-3 DHA and EPA conjugates exert antiproliferative effects in breast cancer cells by inducing autophagy and apoptosis through PPARγ, our findings suggest their potential use as breast cancer preventive and/or therapeutic agents.

INTRODUCTION

Polyunsaturated fatty acids (PUFAs), for long time solely considered of as an energy source in our bodies, have been proven to be highly active molecules. They can act as membrane components able to regulate the fluidity, permeability, and dynamics of cell membranes, but also as ligands in signal transduction and as transcription factors modulating protein synthesis (Chapkin et al., 2008). Most but not all fatty acids can be synthesized in the human body. In particular, essential fatty acids which are those required for biological processes must be obtained from dietary sources (Williams and Burdge, 2006). The two major family of essential fatty acids are the omega-3 and omega-6 PUFAs, whose ratio in the body is believed to be of higher importance than the absolute levels of a certain fatty acid in cancer incidence (Gleissman et al., 2010). Existing reports suggest that omega-6 essential fatty acids are typically proinflammatory and are linked with initiation and progression of carcinogenesis (Lanson et al., 1990; Cohen, 1997; Chapkin et al., 2007; Hyde and Missailidis, 2009); whereas omega-3 essential fatty acids have broad health benefits, including anticancer properties (Serini et al., 2011 and references therein). Indeed, consumption of the two main omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), naturally present in fish, is associated with decreased risk of breast, prostate, colon, and kidney cancers (Smith-Warner et al., 2006; Wolk et al., 2006; Courtney et al., 2007; Fradet et al., 2009; Thiebaut et al., 2009; West et al., 2010). Antineoplastic activities of n-3 PUFAs include alteration of membrane fluidity and cell surface receptor function, modulation of COX activity, and increased cellular oxidative stress (Calviello et al., 2009). In breast cancer cells, DHA strongly reduces cell viability and DNA synthesis and promotes cell death via apoptosis (Kang et al., 2010); while EPA inhibits mitogen activation of AKT survival pathway and enhances the growth inhibitory response to the antiestrogen tamoxifen (deGraffenried et al., 2003). These findings are consistent with microarray studies revealing that both fatty acids are able to modulate the expression of genes involved in the regulation of apoptosis, defense immunity, and cell growth in several breast cancer cell lines (Hammamieh et al., 2007). Interestingly, the biological effects of DHA and EPA can also be exerted by their

derivative molecules which are widely present in nature and endogenously metabolized in a tissue-specific manner (Brown et al., 2011; Kim et al., 2011).

In the last years, *N*-acyl amines of DHA and EPA and other n-3 PUFAs-derived conjugates with amino acids and neurotransmitters have attracted much attention because of their potential roles in pathophysiological conditions suggesting that they could represent a new additional class of endogenous signaling molecules (Meijerink et al., 2013). Chemically, these compounds are amphiphilic molecules, characterized by a head group that may be charged or neutral and a hydrocarbon tail that allows them to penetrate into cell membranes, potentially affecting their functional properties (Connor et al., 2010). It has been demonstrated that n-3 PUFAs-derived conjugates and their parental molecules show affinity for different receptors, including cannabinoid receptors (CB_1 and CB_2), several G protein-coupled receptors, transient receptor potential channel type V1 (TRPV1), and peroxisome proliferator-activated receptors (PPARs) (Connor et al., 2010 and references therein). Conjugates of n-3 PUFAs with ethanolamines, serotonin, L-alanine, L-serine, histidine, GABA, glutamic acid, or dopamine have been found in mammals (Meijerink et al., 2013 and references therein). The biological activity of these compounds can be regulated by the interaction with different enzymes involved in their metabolism. The half-life of all these endogenous fatty acids amides is very short due to rapid deactivation by fatty acid amide hydrolase (FAAH) (Lambert and Fowler, 2005; Ezzili et al., 2010). Notably, some of n-3 PUFA-derived conjugates, such as *N*-arachidonoyl dopamine, acting as inhibitors of FAAH may have consequences for their metabolic stability (Bisogno et al., 2000). Among DHA- and EPA-conjugates the *N*-acyl-ethanolamines, DHEA and EPEA, show anti-inflammatory or general immune modulating properties (Burstein and Zurier, 2009; Pandey et al., 2009) and possess antitumoral activities in prostate cancer cells (Brown et al., 2010), highlighting the importance of these compounds as potential pharmacological tools. On the other hand, some of *N*-acyl-dopamines, including DHA-dopamine (DA) and EPA-DA, produce cannabinoid-like effects *in vivo* via CB_1 (Bezuglov et al., 2001), exert positive effects on hypoxic-ischemic injury or brain inflammatory processes, and inhibit cancer cell proliferation (Burstein and Salmonsen, 2008; Dang et al., 2011), suggesting *N*-acyl-dopamines as potential anti-inflammatory and antitumor leads.

We have synthetized in our laboratory both *N*-acyl-ethanolamines (DHEA and EPEA) and *N*-acyl-dopamines (DHADA and EPADA) (chemical structures are reported in Figure 16.1) and tested their biological activity in different breast cancer cell lines. Notably, we found that DHEA and EPEA as well as DHADA and EPADA are able to exert cell growth inhibition and to trigger autophagy through modulating the expression of the autophagy-related protein, Beclin-1 (Rovito et al., 2013, 2015). Thus, the capacity of these compounds to provide a means of cancer cell death should be taken into consideration for designing novel therapeutic strategies.

DHA AND EPA CONJUGATES INDUCED AUTOPHAGY: REGULATION OF BECLIN-1

Autophagy is a form of nonapoptotic cell death which is activated in response to various cellular stresses depending on cellular context, cell line, and stimulus. The progression

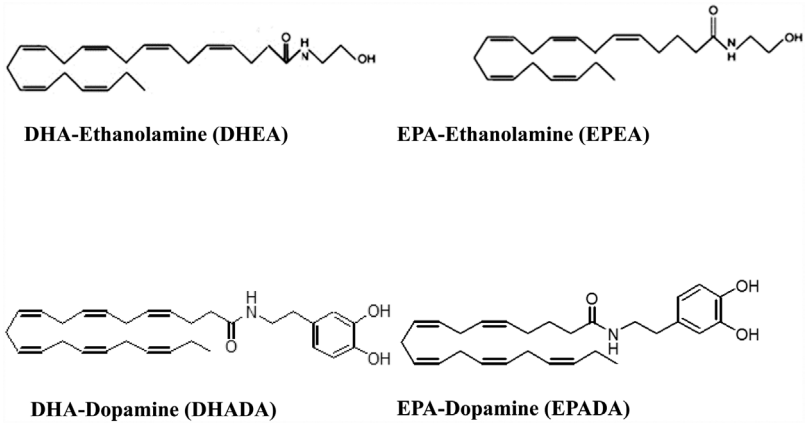

FIGURE 16.1 Chemical structures of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) ethanolamine and dopamine conjugates.

of autophagy is tightly regulated by a group of evolutionarily conserved genes, the *Atg* (autophagy-related) genes, and consists of different stages: initiation, autophagosome formation (nucleation, elongation, and completion), maturation, and degradation, which eventually results in lysosomal breakdown of cytoplasmic material. The initiation of autophagy is controlled by the ULK1 (human homolog of Atg1) kinase complex that integrates stress signals from the mammalian target of rapamycin (mTOR). When mTOR is inhibited, autophagic process can occur and involve vacuolar sorting protein 34 (Vps34), a class III phosphoinositide 3-kinase (PI3K3), that forms a complex with Beclin-1. Beclin-1 is a key protein in autophagy acting as a platform to assemble the PI3KC3 complex leading to autophagosome formation. During this phase the microtubule-associated protein 1 light-chain 3 (LC3), the mammalian ortholog of Atg8, is lipidated by covalent attachment of phosphatidyl ethanolamine, a process mediated by the protein Atg7. Upon completion of autophagosome formation and with the exception of a proportion of LC3 bound to the luminal membrane, the Atg proteins are then recycled in the cytosol. LC3 binds to the adaptor protein p62/sequestosome1 that is involved in trafficking proteins to the proteasome and serves to facilitate the autophagic degradation of ubiquitinated protein aggregates. The late events in autophagy involve the final maturation and fusion of autophagosomes with lysosomes to form an autolysosome leading to a final degradation (Klionsky and Emr 2000; Levine and Klionsky, 2004).

Regulation of Beclin-1 at Nongenomic Level

Beclin-1, the mammalian orthologue of the yeast Atg6, is one of the major proteins related to autophagy. It is expressed in many human tissues and is localized primarily within cytoplasmic structures, including the endoplasmic reticulum, mitochondria, and the perinuclear membrane. Beclin-1 contains three identified structural domains: a Bcl-2 (B-cell lymphoma-2)

homology-3 (BH3) domain (amino acids 114–123) at the N-terminus, a central coiled-coil domain (CCD, amino acids 144–269), and an evolutionarily conserved domain (ECD, amino acids 244–337). The ECD is essential for the ability of Beclin-1 to mediate autophagy and inhibit tumorigenesis. Beclin-1 also contains a short leucine-rich amino acid sequence that is responsible for its efficient nuclear export signal (NES). Mutations of the Beclin-1 NES interfere with its ability to promote nutrient deprivation–induced autophagy and to suppress tumorigenesis (Liang et al., 2001). Beclin-1 has been shown to form a protein complex with Ambra1 (activating molecule in Beclin-1-regulated autophagy), UVRAG (UV radiation resistance-associated gene), and Atg14L (a putative mammalian homolog for yeast Atg14) which interacts with its CCD, and with PI3KC3/Vps34 complex which instead interacts with both ECD and CCD. The CCD, a universal oligomerization domain, mediates Beclin-1 self-interaction and dimer formation *in vivo* and *in vitro* (Adi-Harel et al., 2010; Noble et al., 2008). Most importantly, through its BH3 domain Beclin-1 interacts with the antiapoptotic proteins Bcl-2 and Bcl-X_L (B-cell lymphoma extra long). This interaction results in the inhibition of Beclin-1 autophagic function (Pattingre et al., 2005), suggesting Beclin-1 as a key molecule at the crossroad between autophagic and/or apoptotic cell death. Indeed, the binding of Beclin-1 to the endoplasmic reticulum–localized Bcl-2 reduces starvation-induced autophagy.

There are different means to regulate dissociation of Beclin-1 and Bcl-2 during autophagy in mammals, including the phosphorylation of either one of the two partners in the complex. Beclin-1 could be phosphorylated by death-associated protein kinase on its BH3 domain, and this phosphorylation leads to the dissociation of Beclin-1 from its inhibitors to activate the autophagic machinery (Zalckvar et al., 2009). On the other hand, it has been reported that phosphorylation of Bcl-2 by c-Jun N-terminal kinase 1 (JNK1) at residues threonine 69, serine 70, and serine 87 within the nonstructural loop of Bcl-2 (Wei et al., 2008) or by extracellular signal–regulated kinase (ERK) (Tang et al., 2010) reduces the interaction between Beclin-1 and Bcl-2 thus promoting autophagy.

In this regard, we demonstrated that omega-3 PUFAs ethanolamines DHEA and EPEA increased Bcl-2 phosphorylation at serine 70 reducing its physical association with Beclin-1 thus resulting in the increased levels of Beclin-1 unbound protein in human breast cancer cells, as indicated by immunofluorescence analysis (Figure 16.2).

Conflicting data are reported on the role of phosphorylated Bcl-2. It has been suggested that the phosphorylation of serine 70 inactivates the anti-apoptotic function of Bcl-2 (Yamamoto et al., 1999), while other studies reported that phosphorylation of Bcl-2 at serine 70 site would enhance its anti-apoptotic functions (Ito et al., 1997).

In our experimental model, DHEA and EPEA treatments did not induce any changes in the internucleosomal fragmentation profile of genomic DNA, which is a diagnostic hallmark of cells undergoing apoptosis, addressing that DHEA- or EPEA-dependent cell growth inhibition does not occur through an apoptotic process but may involve autophagic pathway (Rovito et al., 2013).

Regulation of Beclin-1 at Genomic Level

Apart from the upregulatory effects exerted by DHA and EPA conjugates with ethanolamine on Beclin-1, our recent data revealed that dopamine conjugates were also able to

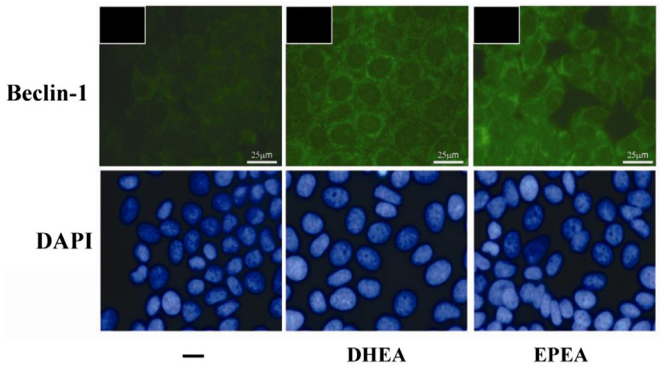

FIGURE 16.2 Upregulation of Beclin-1 expression by DHEA and EPEA in human breast cancer cells, MCF7. Immunofluorescence of Beclin-1 (upper panels) and DAPI (lower panels) in MCF7 cells untreated (–) or treated for 6 h with DHEA or EPEA 1 μM. Small squares, negative controls. Bar, 25 μm.

induce Beclin-1 expression in ER/PR-positive MCF-7, ER/PR double negative and HER2 positive SKBR3, and triple-negative MDA-MB-231 cells. Particularly, the upregulation of Beclin-1 expression, at both protein and mRNA levels, following DHADA and EPADA administration, prompted us to investigate the transcriptional regulation of Beclin-1. Of interest, in breast cancer cells either DHADA or EPADA increased Beclin-1 levels by inducing its gene promoter transcriptional activity (Rovito et al., 2015).

Increasing evidence suggests that several transcription factors are involved in the regulation of *beclin 1* which represents the first identified tumor suppressor gene in human to induce autophagy (Liang et al., 1999). The *beclin 1* gene maps to the human chromosome 17q21, which is monoallelically deleted in 40–75% of human prostate, breast, and ovarian cancers, and Beclin-1 is commonly expressed at reduced levels in those tumors (Aita et al., 1999). The human *beclin 1* gene contains a 1.5 kb CpG island spanning from the promoter to part of the intron 2, suggesting that DNA methylation may be responsible for downregulation of Beclin-1 expression in cancer (Li et al., 2010). In addition, sequence analysis of the promoter region of the *beclin 1* gene (GenBank accession number AY494710) indicated that the proximal promoter region contains binding sites for different transcription factors, such as AP-1, AP2, CREB, nuclear factor kappa B (NF-kB), Sp1, and E2F (Tang, et al. 2009) underlying the existence of different mechanisms involved in *beclin 1* gene regulation. Particularly, *beclin 1* could be an E2F target as suggested by a chromatin immunoprecipitation (ChIP)-on-chip study (Kusama et al., 2009), since this transcription factor is able to directly transactivate the *beclin 1* promoter (Wang et al., 2010). It has been also reported that regulation of Beclin-1 expression may involve microRNAs (miRNAs) (Kang et al., 2011), among which the miR-30a, binding to its specific sequence in the 3′-UTR of *beclin 1*, plays a distinct role. Treatment with a miR-30a antagomir resulted in upregulation of Beclin-1, whereas transfection with a miR-30a mimic decreased Beclin-1 mRNA and protein expression (Zhu et al., 2009), resulting in decreased autophagic activity in cancer cells (Kang et al., 2011). Moreover, p65, one of the canonical NF-kB pathway components, directly binds the Beclin-1 promoter

and upregulates its mRNA and protein levels, leading to autophagy in T cells (Copetti et al., 2009). It is worth noting that the transcription factor binding motifs for NF-kB as well as for Sp1 and RXR within the *beclin 1* promoter are also potential PPARγ binding sequences (Mangelsdorf and Ronald, 1995; Bonofiglio et al., 2006, 2008).

Our findings revealed that DHADA and EPADA upregulated *beclin 1* transcriptional activity acting as PPARγ activators. Particularly, in transient transfection experiments using Beclin-1 promoter deleted constructs, the responsiveness to DHADA and EPADA was no longer noticeable in the absence of the RXR sequence closer to the initiation transcription site, suggesting that this RXR site is required for *beclin 1* promoter activation by both conjugates. Besides, the involvement of either PPARγ and/or RXR in the stimulatory effect of DHADA and EPADA was evidenced by the ability of the PPARγ antagonist GW as well as the RXR antagonist UVI to abolish the Beclin-1 promoter activation. Furthermore, the physiological relevance of RXR site, as it exists in native chromatin, has been proved by ChIP and Re-ChIP assays by using anti-RXR and/or anti-PPARγ antibodies. Interestingly, PPARγ/RXR occupancy of the RXR site containing region of *beclin 1* promoter was induced in a DHADA/EPADA-dependent manner, since recruitment of RXR and/or PPARγ was enhanced by both conjugates. These events were concomitant with an increased association of RNA-polymerase II to the *beclin 1* promoter and consistent with the upregulation of *beclin 1* gene expression (Rovito et al., 2015). All together our findings demonstrated a novel mechanism by which DHA and EPA conjugates by modulating Beclin-1 expression at either nongenomic or genomic level may regulate autophagy (Rovito et al., 2013, 2015).

AUTOPHAGIC PROCESS TRIGGERED BY DHA AND EPA CONJUGATES

Autophagy can have dual roles in cancer by either suppressing tumor growth or promoting cell survival, thus understanding autophagy regulation during different stages of cancer development and progression and identifying the involved cellular signaling pathways it is essential for its optimal exploitation for therapeutic advantage. Several signaling pathways are involved in the regulation of autophagy in mammalian cells, comprising PI3K/Akt/mTOR, which acts through mTOR kinase as an important repressor of autophagy, MAPK/MEK/ERK which regulates the maturation of autophagosomes, and p38 which inhibits autophagy by antagonizing the ERK pathway (Corcelle et al., 2007; Comes et al., 2007; Chiacchiera and Simone, 2008; Yu et al., 2010). Autophagy is a catabolic degradation process whereby cellular proteins and organelles are engulfed into autophagosomes, digested in lysosomes, and recycled to sustain cellular metabolism. Accurate identification of the various autophagic organelles is difficult, and it is also very hard to determine whether observed autophagosome-like structures represent sealed entities or not. By far the most widely used approach to monitor the autophagic process in mammalian cells involves the autophagy-related marker LC3 (Seglen et al., 2015). This protein is linked to the phagophoric membrane by lipidation during the final steps of phagophore assembly, and it remains associated with autophagic organelles until it is degraded in the lysosomes. Moreover, autophagolysosome formation can be easily and accurately monitored by measuring the fluorescence of

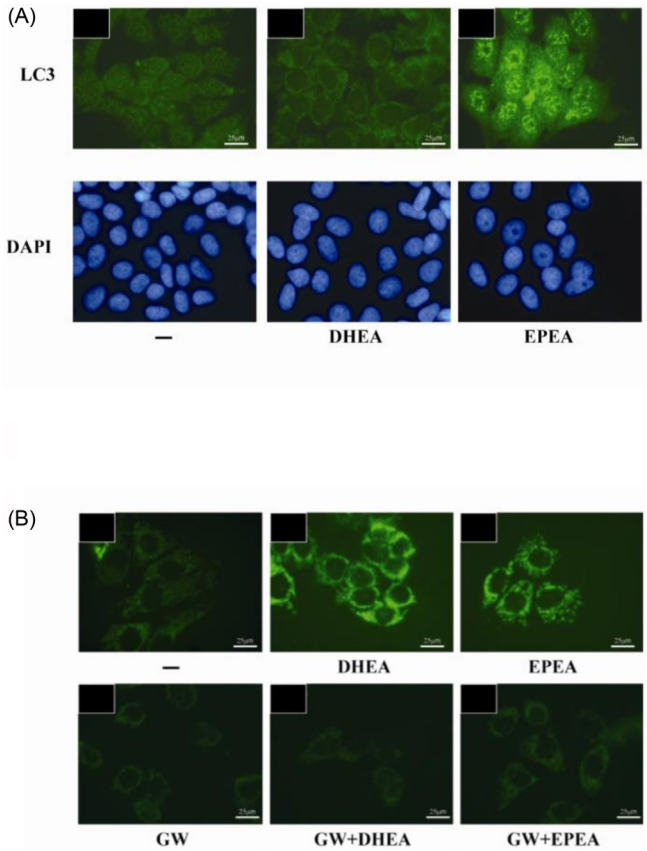

FIGURE 16.3 PPARγ-mediated autophagy by DHEA and EPEA in human breast cancer cells, MCF-7. (A) Immunofluorescence of microtubule-associated protein 1 light chain 3 (LC3) (upper panels) and DAPI (lower panels) in MCF-7 cells untreated (−) or treated for 6 h with DHEA or EPEA 1 μM. (B) Monodansylcadaverine staining of cells untreated (−) or treated for 12 h with DHEA or EPEA 1 μM. Small squares, negative controls. Bar, 25 μm.

the drug monodansylcadaverine (MDC) incorporated by the cells under different experimental conditions (Biederbick et al., 1995).

Taking advantage of these methodological approaches, the ability of DHA and EPA ethanolamine and dopamine conjugates to trigger the autophagic process in breast cancer cells has been investigated along with evaluation of phosphorylation levels of p38 showing that their administration reduced phosphorylation state of p38, enhanced formation of autophagosomes, and significantly increased LC3 immunofluorescence (Figure 16.3) (Rovito et al., 2013). Interestingly, PPARγ is involved in autophagy induced by DHA and EPA conjugates autophagy as evidenced by the ability of the PPARγ antagonist GW to reduce LC3 expression and to prevent the accumulation of MDC-labeled vacuoles (Rovito et al., 2013, 2015) (Figure 16.3). These observations are consistent with the previous described

modulation of the autophagic protein Beclin-1 by PPARγ, addressing this receptor as a key factor in the determinism of cell fate.

PPARγ AS CENTRAL MEDIATOR IN THE CROSS TALK BETWEEN AUTOPHAGY AND APOPTOSIS

All our findings indicate that DHEA and EPEA as well as DHADA and EPADA treatment induces cell death by autophagy in a PPARγ-dependent manner in breast cancer cells. PPARγ is a member of the nuclear receptor family of ligand-dependent transcription factors, which is best known for its differentiating effects on adipocytes and insulin-mediated metabolic functions. PPARγ is also involved in cell cycle control, inflammation, atherosclerosis, apoptosis, and carcinogenesis (Desvergne and Wahli, 1999). Most published studies imply the inhibitory effects of PPARγ ligands on the tumor growth of several carcinomas, including breast cancer (Elstner et al., 1998; Grommes et al., 2004). In the past few years, we have investigated different molecular mechanisms through which PPARγ may induce antiproliferative effects and cell cycle arrest in human breast cancer cells (Bonofiglio et al., 2005, 2006). Besides, in these cells as emerged from many current lines of evidences a cross talk between PPARγ activity and death signaling pathways does exist leading to apoptosis and autophagy (Bonofiglio et al., 2009a,b; Schmidt et al., 2010; Rovito et al., 2013).

Stemming from the ability of PPARγ to regulate the transcription of *phosphatase and tensin homolog on chromosome ten* (*PTEN*) (Patel et al., 2001) which is associated with a suppression of AKT–mTOR signaling pathway, we found that DHEA and EPEA were able to upregulate PTEN expression and to inhibit its downstream AKT–mTOR pathway suggesting that both compounds acting as PPARγ ligands may trigger either apoptotic or autophagic processes (Rovito et al., 2013).

In the complex interplay existing between autophagy and apoptosis, autophagic process may precede apoptosis (Maiuri et al., 2007). It has been reported that among autophagy protein fragments resulting from caspases cleavage, proteolytic form of Beclin-1 localizes to mitochondria and causes the release of cytochrome C which, representing a critical step in the apoptotic cascade, facilitates apoptosis (Wirawan et al., 2010).

Our data showed, following a short-term (24 h) exposure to DHEA and EPEA as well as to DHADA and EPADA, the absence of both poly (ADP-ribose) polymerase (PARP) cleavage and DNA laddering which are the typical hallmarks of cells undergoing apoptosis, confirming that in this experimental conditions autophagy is the first early event governing breast cancer cell death (Rovito et al., 2013, 2015). Instead, prolonged treatment (48 h) with DHADA and EPADA significantly reduced Beclin-1 expression, enhanced proapoptotic Bid levels, caused the release of cytochrome C from mitochondria into the cytosol, and induced PARP cleavage. Moreover, also DNA laddering revealed changes in the internucleosomal fragmentation profile of genomic DNA in breast cancer cells treated with DHA and EPA conjugates. Thus, overall our results demonstrated that upon DHADA and EPADA exposure autophagy precedes apoptotic cell death in breast cancer cells (Rovito et al., 2015). In conclusion, our findings provided new insights into the molecular mechanism through which PPARγ, as central player in the cross talk between autophagy and apoptosis, mediates DHADA- and EPADA-induced cell death in breast cancer cells.

DISCUSSION

Dietary factors and particularly dietary lipids have long been studied for their association with breast cancer risk, survival, and recurrence (Glade, 1999; Rock and Demark-Wahnefried, 2002; Bougnoux et al., 2010). Increased dietary consumption of the long chain n-3 PUFA, DHA, and EPA have been demonstrated to inhibit breast carcinogenesis by decreasing cancer cell viability, proliferation, invasion and by increasing chemosensitivity (Evans and Hardy, 2010). Recently, a great deal of excitement has been generated by the identification of a series of *N*-acylamines of n-3 PUFAs acting as endogenous mediators whose biological significance, however, still remains to be fully established. Although diet is known to be one of the main environmental factor modulating the formation of these derivative compounds, the association between dietary intake of n-3 PUFAs and their synthesis as well as their role and significance in mediating the alleged health effects of fish oil remain speculative. Interestingly, in recent studies some of these molecules have been proved to display a more potent anticancer efficacy and potency compared with parent fatty acids which, together with their ability to inhibit FAAH thus increasing their (local) concentrations (Meijerink et al., 2013), makes them of interest to both the pharmacological and nutritional research fields. Evidence is accumulating that among n-3 PUFAs-derived *N*-acylamines, *N*-acyl ethanolamines and *N*-acyl dopamines possess several properties (Brown et al., 2011; Burstein and Zurier, 2009; Dang et al., 2011) that deserve further research in relation not only to inflammatory and neural disorders, but also to cancer.

We have shown, for the first time, that DHEA and EPEA as well as DHADA and EPADA inhibit breast cancer cell growth, whereas they do not affect growth of nontumorigenic breast epithelial cells. The molecular mechanism underlying this biological effect requires the involvement of PPARγ activation (Rovito et al., 2013, 2015).

Previous findings demonstrated that n-3 PUFAs and their derivatives act as natural ligands of PPARγ which mediates their effects on cell function (Wolfrum et al., 2001; Tan et al., 2002; Hihi et al., 2002; Allred et al., 2008). Accordingly, our studies evidenced that in breast cancer cells both conjugates, while decreasing breast cancer cell proliferation, are able to activate the endogenous PPARγ and to upregulate its mRNA and protein levels, enhancing the expression of a PPARγ target gene such as the tumor-suppressor PTEN. In turn, increased PTEN cellular content determined suppression of AKT–mTOR survival signaling pathway contributing to trigger autophagic process in breast cancer cells (Rovito et al., 2013).

Autophagy is an essential process that consists of selective degradation of cellular components. The initial step of autophagy is regulated by activation of class III PI3K in a complex with autophagy-associated protein Beclin-1 (Levine and Deretic, 2007). Beclin-1 was originally discovered in a yeast two-hybrid screen as a Bcl-2-interacting protein and was the first human protein shown to be indispensable for autophagy (Liang et al., 1999).

Interestingly, DHEA and EPEA acting as PPARγ ligands induce phosphorylation of Bcl-2 on serine 70 promoting its dissociation from Beclin-1 whose cellular available protein levels result thereby increased (Rovito et al., 2013). According to the antiapoptotic functions of phosphorylated Bcl-2 (Ito et al., 1997), in our experimental model, short-term exposure of breast cancer cells to DHEA and EPEA did not induce any changes in the internucleosomal fragmentation profile of genomic DNA, suggesting that the early DHEA-/EPEA-dependent growth inhibition does not occur through an apoptotic process, but may involve

the autophagic pathway (Rovito et al., 2013). Noteworthy, from our findings it emerges a dual mechanism inducing autophagy: DHEA and EPEA administration causes the increase of unbound Beclin-1 fraction within the cell, while DHADA and EPADA modulation of Beclin-1 cellular content occurs at the transcriptional level and is mediated by PPARγ (Rovito et al., 2015). Activation of PPARγ is a multistep process that involves ligand binding, heterodimerization with RXR, interaction with cognate DNA sequences, and recruitment of coregulatory proteins (Mangelsdorf et al., 1995). Our findings showed that transactivation of Beclin-1 promoter by DHADA and EPADA directly involves recruitment of either PPARγ or RXR together with RNA polymerase II leading to enhanced Beclin-1 gene transcriptional activity (Rovito et al., 2015).

Regardless whether the mechanism leading to the rise of Beclin-1 is direct or indirect, induction of Beclin-1 expression in breast cancer cells upon short-term administration of DHA and EPA conjugates causes autophagy, since it leads to the formation of autophagolysosome with the involvement of proteins which exert pivotal roles in the autophagic cell death. Among them, LC-3 protein, which is a specific membrane marker for the detection of early autophagosome formation, is enhanced in cells treated with both ethanolamine or dopamine conjugates. Autophagy occurrence is also documented by the concomitant marked increase of autophagic vacuoles formation, as emerged by MDC fluorescence (Rovito et al., 2013, 2015).

Distinct modes of cell death can occur. Although initially thought to represent mutually exclusive cellular states, it has recently shown the existence of cellular context that necessitates a balanced cross talk between autophagy and apoptosis. Indeed, these two modes of cellular cell death are often regulated by similar signaling pathways, engaged common subcellular sites and organelles, and even share initiator and effector molecules (Nikoletopoulou et al., 2013). In this scenario, Beclin-1 appears to be a fundamental factor at the crossroad between autophagy and apoptosis. Under sustained stress conditions, caspase-mediated cleavage of Beclin-1 abrogates the autophagic function of Beclin-1(Wirawan et al., 2010). Importantly, the C-terminal fragment that results from this cleavage acquires a new function and following its delocalization to the mitochondria can amplify mitochondrion-mediated apoptosis, by sensitizing cells to apoptotic signals (Djavaheri-Mergny et al., 2010).

In our experimental models, long-term treatment with DHADA and EPADA triggers apoptotic-related cell death as indicated by increased proapoptotic levels of Bid and the release of cytochrome C, concomitant with PARP cleavage leading to changes in the internucleosomal fragmentation profile of genomic DNA (Rovito et al., 2015).

In conclusion, we have provided new insights into the molecular mechanism by which DHA and EPA conjugates through PPARγ activation upregulate Beclin-1 expression in a transcriptional-independent and -dependent manner promoting autophagy in breast cancer cells (Figure 16.4). Moreover, our findings highlight the role of PPARγ as a central molecule in the cross talk between autophagy and apoptosis able to mediate cell death induced by DHEA and EPEA as well as DHADA and EPADA in breast cancer cells. Understanding the mechanism of action of dietary components that have preventive and therapeutic effects on cancer is one of the main challenges for modern science. In this context our data emphasize the importance of the two omega-3 PUFA conjugates, ethanolamine- or dopamine conjugates, as new pharmacological tools to be perspectively implemented in the adjuvant therapy for breast cancer treatment.

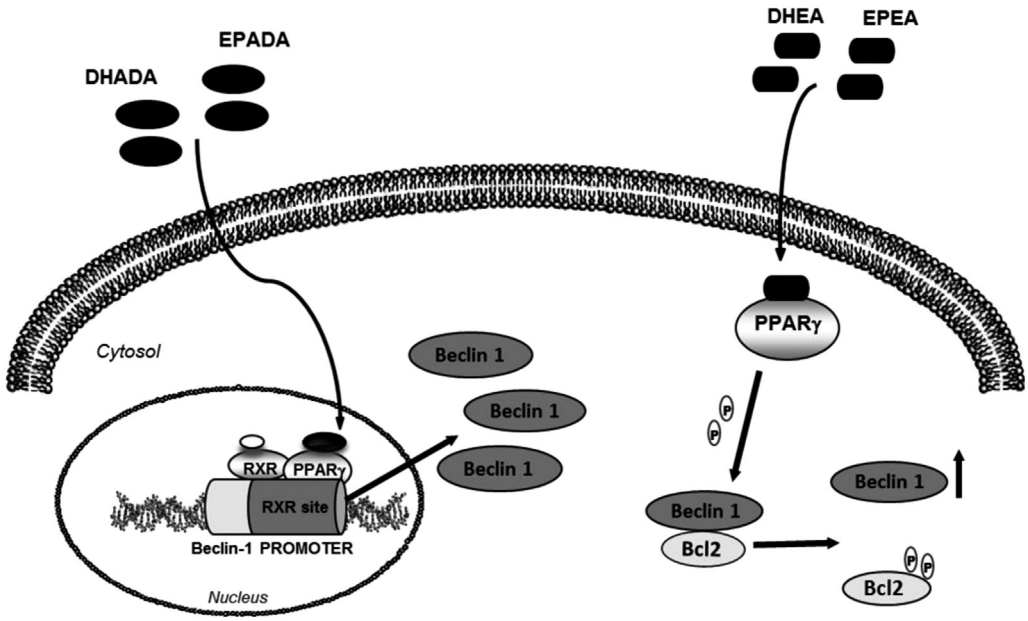

FIGURE 16.4 Hypothetical model of molecular mechanisms by which DHA- and EPA conjugates through PPARγ activation upregulate Beclin-1 expression in a transcriptional-dependent and -independent manner promoting autophagy in breast cancer cells.

References

Adi-Harel, S., Erlich, S., Schmukler, E., et al., 2010. Beclin 1 self-association is independent of autophagy induction by amino acid deprivation and rapamycin treatment. J. Cell. Biochem. 110, 1262–1271.

Aita, V.M., Liang, X.H., Murty, V.V., et al., 1999. Cloning and genomic organization of Beclin 1, a candidate tumor suppressor gene on chromosome 17q21. Genomics 59, 59–65.

Allred, C.D., Talbert, D.R., Southard, R.C., et al., 2008. PPARgamma1 as a molecular target of eicosapentaenoic acid in human colon cancer (HT-29) cells. J. Nutr. 138, 250–256.

Bezuglov, V., Bobrov, M., Gretskaya, N., et al., 2001. Synthesis and biological evaluation of novel amides of polyunsaturated fatty acids with dopamine. Bioorg. Med. Chem. Lett. 11, 447–449.

Biederbick, A., Kern, H.F., Elsasser, H.P., 1995. Monodansylcadaverine (MDC) is a specific *in vivo* marker for autophagic vacuoles. Eur. J. Cell Biol. 66, 3–14.

Bisogno, T., Melck, D., Bobrov, M.Y., et al., 2000. *N*-acyl-dopamines: novel *vivo*. Biochem. J. 351, 817–824.

Bonofiglio, D., Gabriele, S., Aquila, S., et al., 2005. Estrogen receptor alpha binds to peroxisome proliferator-activated receptor (PPAR) response element and negatively interferes with PPARgamma signalling in breast cancer cells. Clin. Cancer Res. 11, 6139–6147.

Bonofiglio, D., Aquila, S., Catalano, S., et al., 2006. Peroxisome proliferator-activated receptor activates p53 gene promoter binding to the nuclear factor-kappa B sequence in human MCF7 breast cancer cells. Mol. Endocrinol. 20, 3083–3092.

Bonofiglio, D., Qi, H., Gabriele, S., et al., 2008. Peroxisome proliferator-activated receptor gamma inhibits follicular and anaplastic thyroid carcinoma cells growth by upregulating p21Cip1/WAF1 gene in a Sp1-dependent manner. Endocr. Relat. Cancer 15, 545–557.

Bonofiglio, D., Gabriele, S., Aquila, S., et al., 2009a. Peroxisome proliferator-activated receptor activates fas ligand gene promoter inducing apoptosis in human breast cancer cells. Breast Cancer Res. Treat. 113, 423–434.

Bonofiglio, D., Cione, E., Qi, H., et al., 2009b. Combined low doses of PPARgamma and RXR ligands trigger an intrinsic apoptotic pathway in human breast cancer cells. Am. J. Pathol. 175, 1270–1280.
Bougnoux, P., Hajjaji, N., Maheo, K., et al., 2010. Fatty acids and breast cancer: sensitization to treatments and prevention of metastatic re-growth. Prog. Lipid Res. 49, 76–86.
Brown, I., Cascio, M.G., Wahle, K.W., et al., 2010. Cannabinoid receptor-dependent and -independent anti-proliferative effects of omega-3 ethanolamides in androgen receptor-positive and -negative prostate cancer cell lines. Carcinogenesis 31, 1584–1591.
Brown, I., Wahle, K.W., Cascio, M.G., et al., 2011. Omega-3 *N*-acylethanolamines are endogenously synthesised from omega-3 fatty acids in different human prostate and breast cancer cell lines. Prostaglandins Leukot. Essent. Fatty Acids 85, 305–310.
Burstein, S., Salmonsen, R., 2008. Acylamido analogs of endocannabinoids selectively inhibit cancer cell proliferation. Bioorg. Med. Chem. 16, 9644–9651.
Burstein, S., Zurier, R., 2009. Cannabinoids, endocannabinoids, and related analogs in inflammation. AAPS J. 11, 109–119.
Calviello, G., Serini, S., Piccioni, E., et al., 2009. Antineoplastic effects of n-3 polyunsaturated fatty acids in combination with drugs and radiotherapy: preventive and therapeutic strategies. Nutr. Cancer 61, 287–301.
Chapkin, R.S., McMurray, D.N., Lupton, J.R., 2007. Colon cancer, fatty acids and anti-inflammatory compounds. Curr. Opin. Gastroenterol. 23, 48–54.
Chapkin, R.S., McMurray, D.N., Davidson, L.A., et al., 2008. Bioactive dietary long-chain fatty acids: emerging mechanisms of action. Br. J. Nutr. 100, 1152–1157.
Chiacchiera, F., Simone, C., 2008. Signal-dependent regulation of gene expression as a target for cancer treatment: inhibiting p38alpha in colorectal tumors. Cancer Lett. 265, 16–26.
Cohen, L.A., 1997. Breast cancer risk in rats fed a diet high in n–6 polyunsaturated fatty acids during pregnancy. J. Natl. Cancer Inst. 89, 662–663.
Comes, F., Matrone, A., Lastella, P., et al., 2007. A novel cell type-specific role of p38alpha in the control of autophagy and cell death in colorectal cancer cells. Cell Death 14, 693–702.
Connor, M., Vaughan, C.W., Vandenberg, R.J., 2010. *N*-acyl amino acids and *N*-acyl neurotransmitter conjugates: neuromodulators and probes for new drug targets. Br. J. Pharmacol. 160, 1857–1871.
Copetti, T., Bertoli, C., Dalla, E., et al., 2009. p65/RelA modulates BECN1 transcription and autophagy. Mol. Cell. Biol. 29, 2594–2608.
Corcelle, E., Djerbi, N., Mari, M., et al., 2007. Control of the autophagy maturation step by the MAPK ERK and p38. Autophagy 3, 57–59.
Courtney, E.D., Matthews, S., Finlayson, C., et al., 2007. Eicosapentaenoic acid (EPA) reduces crypt cell proliferation and increases apoptosis in normal colonic mucosa in subjects with a history of colorectal adenomas. Int. J. Colorectal Dis. 22, 765–776.
Dang, H.T., Kang, G.J., Yoo, E.S., et al., 2011. Evaluation of endogenous fatty acid amides and their synthetic analogues as potential anti-inflammatory leads. Bioorg. Med. Chem. 19, 1520–1527.
DeGraffenried, L.A., Friedrichs, W.E., Fulcher, L., et al., 2003. Eicosapentaenoic acid restores tamoxifen sensitivity in breast cancer cells with high Akt activity. Ann. Oncol. 14, 1051–1056.
Desvergne, B., Wahli, W., 1999. Peroxisome proliferator-activated receptors: nuclear control of metabolism. Endocr. Rev. 20, 649–688.
Djavaheri-Mergny, M., Maiuri, M.C., Kroemer, G., 2010. Cross talk between apoptosis and autophagy by caspase-mediated cleavage of Beclin 1. Oncogene 29, 1717–1719.
Elstner, E., Müller, C., Koshizuka, K., et al., 1998. Ligands for peroxisome proliferator-activated receptor and retinoic acid receptor inhibit growth and induce apoptosis of human breast cancer cells *in vitro* and in BNX mice. Proc. Natl. Acad. Sci. U. S. A. 95, 8806–8811.
Evans, L.M., Hardy, R.W., 2010. Optimizing dietary fat to reduce breast cancer risk: are we there yet? Open Breast Cancer J. 2, 108–122.
Ezzili, C., Otrubova, K., Boger, D.L., 2010. Fatty acid amide signaling molecules. Bioorg. Med. Chem. Lett. 20, 5959–5968.
Fradet, V., Cheng, I., Casey, G., et al., 2009. Dietary omega-3 fatty acids, cyclooxygenase-2 genetic variation, and aggressive prostate cancer risk. Clin. Cancer Res. 15, 2559–2566.
Glade, M.J., 1999. Food, nutrition, and the prevention of cancer: a global perspective. American Institute for Cancer Research/World Cancer Research Fund, American Institute for Cancer Research, 1997. Nutrition 15, 523–526.

Gleissman, H., Johnsen, J.I., Kogner, P., 2010. Omega-3 fatty acids in cancer, the protectors of good and the killers of evil? Exp. Cell Res. 316, 1365–1373.
Grommes, C., Landreth, G.E., Heneka, M.T., 2004. Antineoplastic effects of peroxisome proliferator-activated receptoragonists. Lancet Oncol. 5, 419–429.
Hammamieh, R., Chakraborty, N., Miller, S.A., et al., 2007. Differential effects of omega-3 and omega-6 fatty acids on gene expression in breast cancer cells. Breast Cancer Res. Treat. 101, 7–16.
Hihi, A.K., Michalik, L., Wahli, W., 2002. PPARs: transcriptional effectors of fatty acids and their derivatives. Cell. Mol. Life Sci. 59, 790–798.
Hyde, C.A., Missailidis, S., 2009. Inhibition of arachidonic acid metabolism and its implication on cell proliferation and tumour-angiogenesis. Int. Immunopharmacol. 9, 701–715.
Ito, T., Deng, X., Carr, B., et al., 1997. Bcl-2 phosphorylation required for anti-apoptosis function. J. Biol. Chem. 272, 11671–11673.
Kang, K.S., Wang, P., Yamabe, N., et al., 2010. Docosahexaenoic acid induces apoptosis in MCF-7 cells *in vitro* and *in vivo* via reactive oxygen species formation and caspase 8 activation. PLoS One 5, e10296.
Kang, R., Zeh, H.J., Lotze, M.T., et al., 2011. The Beclin 1 network regulates autophagy and apoptosis. Cell Death Differ. 18, 571–580.
Kim, H.-Y., Moon, H.-S., Cao, D., et al., 2011. *N*-docosahexaenoylethanolamide promotes development of hippocampal neurons. Biochem. J. 435, 327–336.
Klionsky, D.J., Emr, S.D., 2000. Autophagy as a regulated pathway of cellular degradation. Science 290, 1717–1721.
Kusama, Y., Sato, K., Kimura, N., et al., 2009. Comprehensive analysis of expression pattern and promoter regulation of human autophagy-related genes. Apoptosis 14, 1165–1175.
Lambert, D.M., Fowler, C.J., 2005. The endocannabinoid system: drug targets, lead compounds, and potential therapeutic applications. J. Med. Chem. 48, 5059–5087.
Lanson, M., Bougnoux, P., Besson, P., et al., 1990. N-6 polyunsaturated fatty acids in human breast carcinoma phosphatidylethanolamine and early relapse. Br. J. Cancer 61, 776–778.
Levine, B., Deretic, V., 2007. Unveiling the roles of autophagy in innate and adaptive immunity. Nat. Rev. Immunol. 7, 767–777.
Levine, B., Klionsky, D.J., 2004. Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev. Cell 6, 463–477.
Li, Z., Chen, B., Wu, Y., et al., 2010. Genetic and epigenetic silencing of the Beclin 1 gene in sporadic breast tumors. BMC Cancer 10, 98.
Liang, X.H., Jackson, S., Seaman, M., et al., 1999. Induction of autophagy and inhibition of tumorigenesis by Beclin 1. Nature 402, 672–676.
Liang, X.H., Yu, J., Brown, K., et al., 2001. Beclin 1 contains a leucine-rich nuclear export signal that is required for its autophagy and tumor suppressor function. Cancer Res. 61, 3443–3449.
Maiuri, M.C., Zalckvar, E., Kimchi, A., et al., 2007. Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nat. Rev. Mol. Cell Biol 8, 741–752.
Mangelsdorf, D.J., Ronald, E.M., 1995. The RXR heterodimers and orphan receptors. Cell 83, 841–850.
Mangelsdorf, D.J., Thummel, C., Beato, M., et al., 1995. The nuclear receptor superfamily: the second decade. Cell 83, 835–839.
Meijerink, J., Balvers, M., Witkamp, R., 2013. *N*-Acyl amines of docosahexaenoic acid and other n-3 polyunsatured fatty acids—from fishy endocannabinoids to potential leads. Br. J. Pharmacol. 169, 772–783.
Nikoletopoulou, V., Markaki, M., Palikaras, K., et al., 2013. Crosstalk between apoptosis, necrosis and autophagy. Biochim. Biophys. Acta 1833, 3448–3459.
Noble, C.G., Dong, J.M., Manser, E., et al., 2008. Bcl-xL and UVRAG cause a monomer-dimer switch in Beclin 1. J. Biol. Chem. 283, 26274–26282.
Pandey, R., Mousawy, K., Nagarkatti, M., et al., 2009. Endocannabinoids and immune regulation. Pharmacol. Res. 60, 85–92.
Patel, L., Pass, I., Coxon, P., et al., 2001. Tumor suppressor and anti-inflammatory actions of PPARgamma agonist are mediated via up-regulation of PTEN. Curr. Biol. 11, 764–768.
Pattingre, S., Tassa, A., Qu, X., et al., 2005. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell 122, 927–939.
Rock, C.L., Demark-Wahnefried, W., 2002. Nutrition and survival after the diagnosis of breast cancer: a review of the evidence. J. Clin. Oncol. 20, 3302–3316.

Rovito, D., Giordano, C., Vizza, D., et al., 2013. Omega-3 PUFA ethanolamides DHEA and EPEA induce autophagy through PPARγ activation in MCF-7 breast cancer cells. J. Cell. Physiol. 228, 1314–1322.
Rovito, D., Giordano, C., Plastina, P., et al., 2015. Omega-3 DHA- and EPA-dopamine conjugates induce PPARγ-dependent breast cancer cell death through autophagy and apoptosis. Biochim. Biophys. Acta. 1850, 2185–2195.
Schmidt, M.V., Brune, B., von Knethen, A., 2010. The nuclear hormone receptor PPARgamma as a therapeutic target in major diseases. Sci. World J. 10, 2181–2197.
Seglen, P.O., Luhr, M., Mills, I.G., et al., 2015. Macroautophagic cargo sequestration assays. Methods 75, 25–36.
Serini, S., Fasano, E., Piccioni, E., et al., 2011. Dietary n-3 polyunsaturated fatty acids and the paradox of their health benefits and potential harmful effects. Chem. Res. Toxicol. 24, 2093–2105.
Smith-Warner, S.A., Spiegelman, D., Ritz, J., et al., 2006. Methods for pooling results of epidemiologic studies: the pooling project of prospective studies of diet and cancer. Am. J. Epidemiol. 63, 1053–1064.
Tan, N.S., Shaw, N.S., Vinckenbosch, N., et al., 2002. Selective cooperation between fatty acid binding proteins and peroxisome proliferator-activated receptors in regulating transcription. Mol. Cell. Biol. 22, 5114–5127.
Tang, D., Kang, R., Livesey, K.M., et al., 2010. Endogenous HMGB1 regulates autophagy. J. Cell. Biol. 190, 881–892.
Tang, H., Da, L., Mao, Y., et al., 2009. Hepatitis B virus X protein sensitizes cells to starvation-induced autophagy via up-regulation of Beclin 1 expression. Hepatology 49, 60–71.
Thiebaut, A.C., Chajes, V., Gerber, M., et al., 2009. Dietary intakes of omega-6 and omega-3 polyunsaturated fatty acids and the risk of breast cancer. Int. J. Cancer 124, 924–931.
Wang, B., Ling, S., Lin, W.C., 2010. 14-3-3 Tau regulates Beclin 1 and is required for autophagy. PLoS One 5, e10409.
Wei, Y., Pattingre, S., Sinha, S., et al., 2008. JNK1-mediated phosphorylation of Bcl-2 regulates starvation-induced autophagy. Mol. Cell 30, 678–688.
West, N.J., Clark, S.K., Phillips, R.K., et al., 2010. Eicosapentaenoic acid reduces rectal polyp number and size in familial adenomatous polyposis. Gut 59, 918–925.
Williams, C.M., Burdge, G., 2006. Long-chain n-3 PUFA: plant v. marine sources. Proc. Nutr. Soc. 65, 42–50.
Wirawan, E., Vande Walle, L., Kersse, K., et al., 2010. Caspase-mediated cleavage of Beclin-1 inactivates Beclin-1-induced autophagy and enhances apoptosis by promoting the release of proapoptotic factors from mitochondria. Cell Death Dis. 1, e18.
Wolfrum, C., Borrmann, C.M., Borchers, T., et al., 2001. Fatty acids and hypolipidemic drugs regulate peroxisome proliferator-activated receptors alpha- and gamma-mediated gene expression via liver fatty acid binding protein: a signaling path to the nucleus. Proc. Natl. Acad. Sci. U.S.A. 98, 2323–2328.
Wolk, A., Larsson, S.C., Johansson, J.E., et al., 2006. Long-term fatty fish consumption and renal cell carcinoma incidence in women. JAMA 296, 1371–1376.
Yamamoto, K., Ichijo, H., Korsmeyer, S.J., 1999. BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G2/M. Mol. Cell. Biol. 19, 8469–8478.
Yu, L., McPhee, C.K., Zheng, L., et al., 2010. Termination of autophagy and reformation of lysosomes regulated by mTOR. Nature 465, 942–946.
Zalckvar, E., Berissi, H., Eisenstein, M., et al., 2009. Phosphorylation of Beclin-1 by DAP-kinase promotes autophagy by weakening its interaction with Bcl-2 and Bcl-X_L. Autophagy 5, 720–722.
Zhu, H., Wu, H., Liu, X., et al., 2009. Regulation of autophagy by a Beclin 1-targeted microRNA, miR-30a, in cancer cells. Autophagy 5, 816–823.

CHAPTER

17

Pro-oxidative Phytoagents Induce Autophagy in Tumors: Villain or Benefactor in Cancer Treatment?

Peng-Nian Yew, Kan-Fu Oh and Wai-Leng Lee

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Abstract

Cancer remains one of the major causes of human death worldwide, especially with recurring, metastatic, or drug-resistant tumors. As botanical preparations have been used for treating various human diseases throughout history, plant-derived chemicals (phytoagents) thus become an important resource for discovery of novel therapeutic agent for cancer treatment. Increasing evidence showed transformed state of cancer confers an altered redox condition that makes cancer cells vulnerable to treatments which augment oxidative stress in the cells. Therefore, studies have shed light on the pro-oxidant effects of some phytoagents pose selective cytotoxicity on cancer cells. In response to oxidative stress stimulated by cytotoxic agents, autophagy is a common cellular process induced to recycle damaged proteins/organelles as a survival mechanism in cells or to trigger type II programmed cell death when the damage is irreparable. In this review, we enumerated several phytoagents reported to induce oxidative stress that inhibit tumor growth. In most cases, anticancer activities of these pro-oxidative phytoagents involve autophagy that provokes apoptosis-independent program, complementing apoptotic cell death to enhance the killing effect. However, some phytoagents induce oxidative stress that stimulates protective autophagy, attenuating their anticancer effect. Therefore, the possible mechanisms on how phytoagent-induced autophagy decides treated cell fate are briefly discussed in this review.

INTRODUCTION

In various types of mammalian cells, autophagy is an important housekeeping process to maintain homeostasis via preventing excessive accumulation of damaged proteins/worn organelles in cells. On the other hand, cells develop redox regulatory system containing antioxidant enzymes and metabolites to resist oxidative stress that is harmful to normal cell function via posttranslational modification of thiol-containing proteins that serve as both important biomolecules and signal mediators (Filomeni et al., 2015). A growing amount of studies demonstrate the important role of autophagy to degrade harmful oxidized molecules in response to oxidative stress, occurring via p62/keap1/Nrf2 pathway (Komatsu et al., 2010). Ubiquitin–proteasome system (UPS) is another degradation machinery that plays an important role in the process (Hao et al., 2013). Abnormal accumulation of oxidized molecules including misfolded protein causes stress in endoplasmic reticulum (ER) which activates interplay between autophagy and UPS to effectively restore homeostatic proteome in the cells (Harding et al., 2002). In transformed cancer cells, reactive oxygen species (ROS) is required to support aggressive proliferative activities and thus increased oxidative basal level in advanced tumors (Schumacker, 2006), making them a group of hyperactive cells vulnerable to additional ROS input or stimuli that suppress protein degradation.

Emerging evidence suggests autophagy a target for cancer treatment. Many studies are exploring potential of both activator and inhibitor of autophagy in treating early and late stages of cancers, respectively (Amaravadi et al., 2011). On the other hand, many therapeutic drugs are known to induce oxidative stress and autophagy in tumors. Currently, drug resistance is a major concern in cancer therapy and inhibition of autophagy was found to sensitize tumor response to certain treatments. Therefore, the clinical use of autophagy inhibitor hydroxychloroquine (HCQ) in sensitizing various cancers is currently being evaluated in several phase I and phase II trials (Duffy et al., 2015). Many phytoagents have also been reported to induce oxidative stress and autophagy in cancer cells. In this review, we firstly introduce the mechanism of autophagy and its role in cancer biology and therapy.

The interconnection between oxidative stress and autophagy is then illustrated in second part of the review. Lastly, autophagy-inducing effects of selected pro-oxidative phytoagents that lead to different cancer cell fate are discussed.

AUTOPHAGY IN CANCER

Overview of Autophagy

Autophagy (eat oneself) is a cellular homeostatic process characterized by catabolic degradation of damaged proteins/organelles for nutrient recycling, and intermittently killing/removing pathogens that are ingested by the cells (Gutierrez et al., 2004; Ogawa et al., 2005). Autophagy activation in response to nutrient deprivation, intracellular/extracellular stress, and DNA damage can temporarily provide nutrients/energy required to maintain cell viability or survival. Generally, autophagy occurs under three conditions: (i) macroautophagy activated by starvation/nutrients deficiency, which involves the fusion of autophagosomes with lysosomes, where sequestered cellular components are digested (Tsujimoto and Shimizu, 2005); (ii) microautophagy with direct engulfment of cytoplasmic material (protein/organelles) into the lysosome through inward folding of lysosome membrane or cellular protrusion; and (iii) chaperone-mediated autophagy (CMA) through the recognition of heat shock chaperon protein HSC70 containing complex, where proteins/organelles with recognition site for HSC70 get unfolded and translocated across lysososme membrane via binding to the chaperone forming CMA-substrate/chaperone complex (Hasima and Ozpolat, 2014).

In autophagy, target of rapamycin (mTOR) macromolecular complex 1 (mTORC1) is the central regulator that serves as a converging point to integrate signals from various pathways, including but not exclusively those pathways which involve AMP-activated protein kinase (AMPK), protein kinase B/Akt (PKB/Akt), phosphatidylinositol-3-kinases (PI3K), p53 mediating transcription, hypoxia inducing factor (HIF), and oxidative stress (Figure 17.1) (Kim et al., 2002; Fang et al., 2001; Alexander et al., 2010; Livesey et al., 2012). In response to various stimuli, signal transductions from these pathways inhibit mTORC1 which then trigger the downstream molecules including a series of autophagy-related proteins (Atgs) to initiate and execute autophagic process in cells. At molecular level, the process of autophagy involves five key steps: (i) induction by stimulus, (ii) initiation of phagophore formation through membrane nucleation, (iii) elongation and maturation of phagophore through processing and insertion of microtubule-associated protein 1 light chain 3 (LC3) into the extending phagophore membrane, (iv) capture and sequestration of targets for degradation, and (v) formation of autophagolysosome and proteolytic degradation of targets by lysosomal proteases (Glick et al., 2010).

Molecular Components and Their Regulation in Autophagy

Autophagy inducer inhibits mTORC1, leading to the induction which involves UNC-51-like kinase (ULK) complex members ULK 1/2, Atg13, Atg101, and FIP200. Virtually autophagy begins with membrane nucleation, an essential step to initiate phagophore

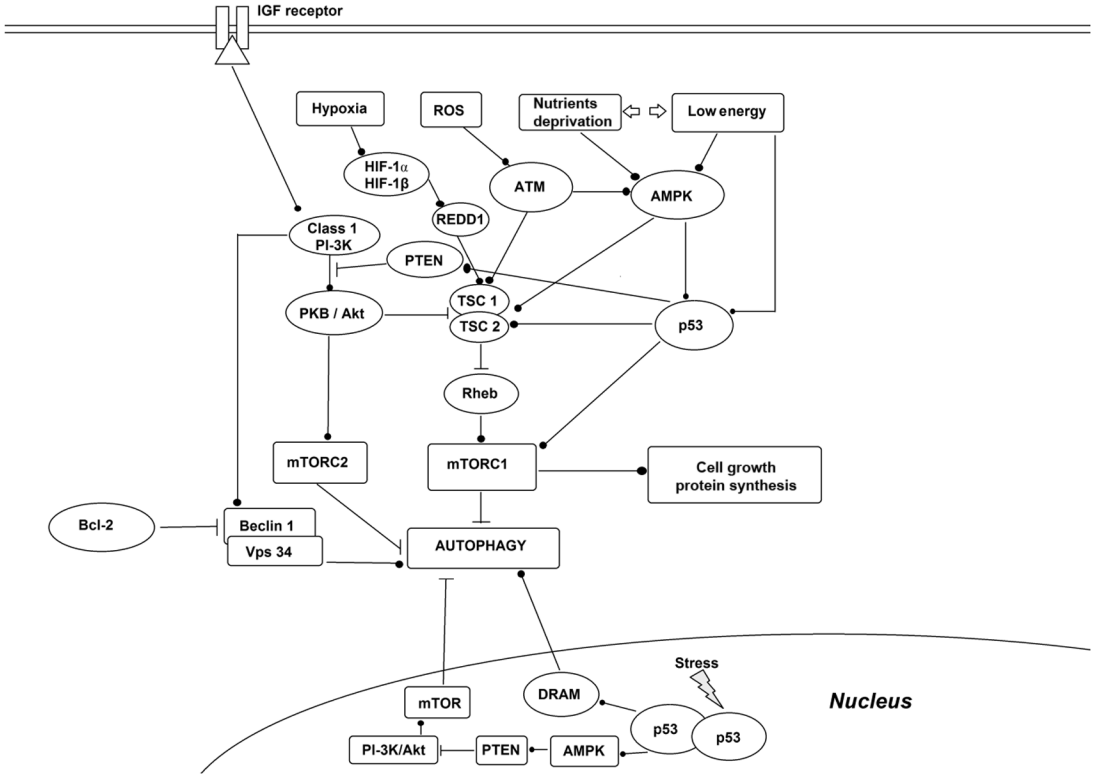

FIGURE 17.1 Schematic overview of signaling pathways regulating autophagy. The common signaling molecules mediating the autophagy include phosphoinositide-3-kinase/protein kinase B (PI3K/Akt), ataxia telangiectasia mutated (ATM), adenosine monophosphate–activated protein kinase (AMPK), and hypoxia-inducible factor 1-alpha (HIF1-α). The mammalian target of rapamycin complex (mTORC) serves as converging point from the AMPK, ATM, PI3K, and HIF stimulation through the TSC1 and TSC2 complex to mediate autophagy and protein synthesis. Stimulation of PIK3/Akt by extracellular growth factor is able to inhibit autophagy through both mTORC1 and mTORC2. On the other hand, the classic pathway of autophagy (involves Beclin-1/Vps34 activation) mediated by the class 1 PI3K activation can facilitate autophagy independent of mTORC. At subside, the antiapoptotic Bcl-2 protein can also prevent autophagy by inhibition of Beclin-1/Vps34. Under chronic nutrient deprivation, the inhibition of cytoplasmic p53 by p53-specific E3 ubiquitin ligase HDM2 is prevented, leading to downstream inhibition of autophagy through mTORC1. In nucleus, genotoxic stress will induce the activation of p53 which induce autophagy through DRAM or the deactivation of PI3K/mTOR inhibition of autophagy by PTEN.

formation regulated by class III PI3K activity and membrane recruitment of Bcl-2 interacting myosin/moesin-like coiled-coil protein 1 (Beclin-1)–Vps34–Vps15 complex. The maturation of phagophore requires ubiquitin-like conjugation systems Atg5–Atg12 and microtubule-associated protein 1 light chain 3 (LC3) conjugation systems. The E1-like enzyme Atg7, E2-like enzyme Atg10, and Atg3 activate both systems, leading to conjugation between Atg12 and Atg5. In the process of target sequestration, the Atg12–Atg5 complex functions as an E3 ubiquitin ligase in catalyzing the conjugation of LC3-I to the lipid phosphatidylethanolamine (PE). On the other hand, Atg5–Atg12 forms complex with Atg16L dimers.

This complex is responsible for membrane curvature of the phagophore by recruiting the LC3B-II. Again, Atg5–Atg12 acting as E3 ligase is essential for lipidation of LC3B-II which lead to the formation of autophagosome. Autophagosome is then fused with lysosome to form an autophagolysosome where the cytosolic macromolecules, proteins, and organelles will be degraded by acid hydrolases (Otomo et al., 2013).

Defects in autophagy could lead to various diseases through alteration of essential metabolic processes in cells. Evidence showed the accumulation of polyubiquitylated protein aggregates and neuronal degeneration in the brain of mice with targeted deletion of Atg5 or Atg7 (Komatsu et al., 2005). Besides, a study indicated the critical role of Atg5 in immunity through maintaining T-cell survival and proliferation (Pua et al., 2007). In comparison to normal tissues, defective autophagic activities were often observed in tumors. An early study using genetically modified mice demonstrated the deletion of Beclin-1 led to tumor formation while reexpression of the protein restored autophagy and inhibited tumor growth, suggesting Beclin-1 is a tumor suppressor (Liang et al., 1999; Yue et al., 2003). By binding to Beclin-1, antiapoptotic proteins from Bcl-2 family were found to inhibit autophagy (Pattingre et al., 2005). In a recent study, inhibition of Bcl-2 triggered autophagic cell death in breast cancer cells (Akar et al., 2008). These studies provided evidences to suggest that the modulation of autophagy in cancer biology could be strategized to prevent or to treat various cancers.

Autophagy in Protein Homeostasis of Cancer

In the process of carcinogenesis, vast varieties of oncoproteins are aggressively expressed in cancer cells. Maintenance of the exuberant cellular activities in cancer cells requires a complex regulatory network for quality control of the oncoproteome. The high rate of protein synthesis and turnover in cancer cells contribute to the vulnerability of these cells to the factors that interfere the proteasomal and autophagosomal protein recycling and degradation machinery (Bruning and Juckstock, 2015). In preservation of metabolic homeostasis, both autophagy–lysosome system and the UPS play important roles in protein turnover. Macroautophagy can degrade all forms of misfolded proteins whereas proteasomal degradation is likely limited to the soluble proteins (Ding and Yin, 2008). Lately, inhibition of the 26S proteasome was found to accumulate nondegraded ubiquitinated proteins which alter cellular bioenergetics, leading to rapid activation of AMPK in cells (Jiang et al., 2015). Activation of AMPK implies trigger of autophagy that could clear the protein aggregates in order to restore cellular homeostasis. Currently most of the findings suggest inhibition of UPS compensatively activates autophagy (Zhu et al., 2010).

In some studies, ER stress was found to regulate the cross talk between the UPS and autophagy machinery. The ER is the major site for synthesis, folding, and modification of cellular protein. Many factors can cause the loss of protein native conformation in ER and generate unspecific aggregations. Uncontrolled aggregation of proteins is proved to be detrimental to normal cellular function. Therefore, misfolded proteins have to be exported out of the ER lumen for degradation by proteasome, a regulation known as ER-associated degradation (ERAD) (Harding et al., 2002). Alternatively, autophagy clears misfolded proteins in response to metabolic stress for instance drug-induced ER stress (Kouroku et al., 2007). Similar to ERAD, ER-associated autophagy (ERAA) is induced in response to accumulation

of misfolded proteins that cause stress in cells which trigger activation of PERK–eIF2α and IRE-1–JNK signaling pathways (Kouroku et al., 2007; Ogata et al., 2006). In this scenario, ERAA is an essential alternative pathway to mitigate the stress caused by misfolded proteins when dysfunction of proteasome occurs to inhibit ERAD.

Modulation of Autophagy in Cancer Treatment: Therapeutic Approach Targeting Cellular Stress Response in Cancer

Autophagy plays different role in various stages of tumorigenesis. In early state of tumor development, induction of autophagy confers protection in cells via removal of carcinogenic damaged proteins and organelles in order to avoid initiation of carcinogenesis (Hanahan and Weinberg, 2011). A wide range of autophagy activators are currently in use for treatment of different cancers. mTOR blocks autophagosome formation in autophagy via preventing Atg13 and ULK1 from forming complex. Thus, inhibition of mTOR is now one of the approaches to stimulate autophagic process in cancer cells. Temsirolimus and everolimus are currently the mTOR inhibitors used in treatment of renal cell carcinoma and breast cancer (Xie et al., 2013). As previously discussed, autophagy can complement the role of UPS when proteasome is dysfunctional in protein degradation. Proteasome inhibitors (PIs) have been shown to activate autophagy and one of the PIs, bortezomib, is used in oncology of multiple myeloma and mantle cell lymphoma. Increased formation of autophagosome and LC3-II was observed in bortezomib-treated cancer cells (Zhu et al., 2010). Another group of autophagy stimulators inhibit histone deacetylase (HDAC) enzymes and accumulate acetylated histones and proteins which increase autophagosome formation. One of the HDAC inhibitors, vorinostat, is used in the treatment of cutaneous T-cell lymphoma, however the details of mechanism is yet to be investigated (Shao et al., 2004). Many tyrosine kinases are found to play important roles in oncogenesis and various tyrosine kinase inhibitors (TKIs) are widely used in anticancer therapy. Imatinib, a therapeutic agent used in malignant glioma treatment, was found to regulate autophagosome induction (Ertmer et al., 2007; Yogalingam and Pendergast, 2008). These compounds induce autophagy beneficial to tumors in early stage however the cytoprotective effects could confer drug resistance in advanced tumor.

As tumor developed into highly malignant stage, autophagy-mediated cytoprotection is in favor of tumor progression under metabolic stress. Therefore, inhibition of autophagy is employed to overcome prosurvival mechanism in late-stage tumors (Degenhardt et al., 2006; Chen and Karantza-Wadsworth, 2009). Currently, lysosomotropic agents are the most widely used autophagy inhibitors. These compounds penetrate lysosomal membrane and get protonated within the vesicles. Accumulation of proton increases pH in lysosome which causes dysfunction in cellular degradation (Kaufmann and Krise, 2007; Yang et al., 2011). Chloroquine (CQ) and HCQ which indirectly modulate late-stage autophagy via suppressing lysosomal degradation are lysosomotropic agents that are used in many preclinical studies (Rosich et al., 2012). Besides protonation in lysosome, CQ inhibits enzymes which acidify lysosomes such as phospholipase A2 and lysophospholipid acylhydrolase (Sotelo et al., 2006). On the other hand, bafilomycin A1 inhibits autophagy indirectly via deregulation of vacuolar-ATPase which acidifies lysosome via proton pumping (Rubinsztein et al., 2012). Different from previously discussed HDAC inhibitor targeting early tumor, Tenovin-6

which inhibits a specific class of HDAC enzyme sirtuin could cause late-stage autophagy (MacCallum et al., 2013). Recently, an ubiquitin-binding HDAC 6 was identified as an essential component of autophagy that targets misfolded proteins and damaged organelles such as mitochondria. It controls the fusion of autophagosomes to lysosomes for effective degradation via remodeling of actin network. Increasing evidence showed inhibition of HDAC 6 leads to accumulation of misfolded proteins and induction of apoptosis in tumor cells through autophagy (Rodriguez-Gonzalez et al., 2008). Despite the effectiveness of these autophagy inhibitors in treating advance tumor, concern remains regarding autophagy-independent properties of these compounds that require further investigation.

Overcoming Drug Resistance via Autophagy Inhibition

Rapid proliferation in tumor increases metabolic demands and generates a myriad of cellular stresses in the process. Robust autophagic activities diminish stress induced damages to sustain tumor progression, implying autophagy a potential target for cancer therapy (Amaravadi et al., 2011). However, a diverse cellular machinery is involved in autophagy and basal level autophagy would generally maintain cell survival. Only when autophagy is overwhelmingly activated then the process will induce apoptosis in targeted cells. Therefore, most of the autophagy modulators would be used in conjunction with other therapeutic agents in clinical implementation. These compounds augment the supplementary effect of autophagy in optimizing killing of cancer cells in the treatment (Chaabane et al., 2013). Duffy et al. summarized 32 ongoing clinical studies evaluating autophagy modulators in various cancer treatments and found more than half of the cases involve lysosomotropic agents: 14 studies used HCQ while 3 studies used CQ. The preliminary phase I and phase II studies provided evidences for the potential therapeutic benefit of autophagy modulators in sensitizing cancers that developed resistance to current cancer therapy (Duffy et al., 2015). As previously discussed, those clinical anticancer drugs targeting early tumor such as mTOR inhibitor, PI, TKI, and HDAC inhibitor could induce autophagy that confer prosurvival effect in highly malignant tumor. Therefore, some of the current phase I trials combined these drugs with HCQ, a lysosomotropic agent which indirectly inhibit autophagy for treatment of a wide range of advanced cancers (Rangwala et al., 2014a; Vogl et al., 2014; Goldberg et al., 2012; Mahalingam et al., 2014).

Lately, ROS formation in chemotherapeutic agents–treated cancer cells was found to play important role in cell death induction (Kaminskyy et al., 2012). ROS can cause damage on most of the macromolecules including protein. Most of the proteins with irreversible oxidative modification should be degraded in order to maintain cellular homeostasis, and UPS is one the important machinery for the purpose (Hao et al., 2013). However, UPS itself is one of the targets of oxidative insults. Overwhelming oxidative stress deactivates proteasome and accumulates oxidatively misfolded proteins which eventually induce cancer cell death (Kriegenburg et al., 2011). Previously we discussed complementary role of autophagy in cellular degradation pathway when UPS is dysfunctional. In good agreement with this scenario, autophagy was shown to protect chemotherapeutic agent–treated cancer cells from ROS-induced threat probably via timely clearance of damaged proteins (Kaminskyy et al., 2012). Therefore, emerging interest is shedding light on the strategy that involves the use of autophagy inhibitors to overcome drug resistance in chemotherapy. Among those

previously described clinical trials, HCQ is also used in combination with chemotherapeutic drugs such as temozolomide and doxorubicin in treatment of glioblastoma multiforme, melanoma, and lymphoma (in pet dogs) (Rosenfeld et al., 2014; Rangwala et al., 2014b; Barnard et al., 2014).

AUTOPHAGY IN ANTICANCER ACTIVITIES OF PRO-OXIDATIVE PHYTOAGENTS

Increasing drug resistance observed in current clinical practice has ignited interest on plant-derived therapeutic substances. Plants produce a wide variety of secondary metabolites (phytochemicals/phytoagents), many of which have been known to reduce risk of cancer development. In our laboratory, a germacranolide sesquiterpene lactone, deoxyelephantopin (DET) was identified from medicinal plant *Elephantopus scaber* (Asteraceas). Firstly, we discovered DET induced ER stress and induced apoptosis in treated mammary carcinoma cells (Lee et al., 2009). Next, we found DET treatment induced ROS, proteasome inhibition, and formation of aggresome in mammary carcinoma cells (Lee and Shyur, 2012). These findings suggest action of DET resembles pro-oxidative agent that induces ROS inhibiting proteasome activity. In addition, formation of aggresome in treated cells implies DET-mediated proteasome inhibition is accompanied by autophagic response. Together these findings are in good agreement with previously discussed scenario: ER-mediated cross talk between proteasome and autophagy and misfolded proteins can cause ER stress. It is reported in a study that PIs activated autophagy as a cytoprotective response in human prostate cancer cells (Zhu et al., 2010). A more recent study indicates autophagy can clear aggresome to mitigate the stress and cause drug resistance (Abdel Malek et al., 2015). Therefore, a clearer picture is now obtained on those therapeutic approaches which involve inhibition of autophagy-mediated aggresome clearance to enhance therapeutic effect of pro-oxidative agent and to overcome drug resistance (Carew et al., 2010; David et al., 2010; Komatsu et al., 2013). In previous section, we introduced the use of lysosomotropic agent such as HCQ widely employed in clinical trials for autophagy inhibition. However, indirect autophagy inhibition by lysosomotropic agent is not specific to cancer cells but applicable to wide range of cell types (Kaufmann and Krise, 2007; Yang et al., 2011). On the other hand, many pro-oxidative phytoagents were proved with specific toxicity to cancer cells and some of these compounds were found to affect autophagy in treated cells. Therefore in the following section, we will discuss the regulatory role of these phytoagents on autophagy and their potential to be used as natural autophagy modulators in cancer treatment.

Oxidative Stress and Autophagy in Cancer Therapy

In the process of tumor progression, ROS signals drive proliferation and many cellular events in cancer cells. Elevated basal oxidative stress compromises defense of advanced tumor against therapeutic agents which generate ROS or attenuate antioxidant system in the cells (Schumacker, 2006). A number of anticancer drugs such as vinblastine, doxorubicin, campthotecin, cisplatin, and inostamycin were found to induce apoptosis in cancer cells via hydrogen peroxide (H_2O_2) generated by activation of NADPH oxidase (Simizu et al., 1998).

On the other hand, enzymes such as catalase, glutathione peroxidase (GPx), and superoxide dismutase (SOD) which play essential roles in cellular antioxidative system are often deficient in cancer cells, and these cells acquire a low tolerance to oxidative stress therefrom. Therefore, "oxidative therapy" which involves delivery of excessive ROS into cancer cells or attenuation of antioxidative system in cancer cells was recently proposed in treatment of advanced cancers (Fang et al., 2009). When antioxidant defenses are surpassed by ROS induction and cause irreversible damage in cells, oxidative stress leads to cell death. A potent anticancer agent gambogic acid was recently reported to induce oxidative stress dependent caspase activation which triggers both apoptosis and autophagy in human bladder cancer cells (Ishaq et al., 2014). Different from the well-characterized apoptosis, there are limited studies to confirm autophagy as the genuine cause of cell death. Most of the studies, which proposed autophagy as a mechanism to execute cell death, were mainly based on unspecific action of pharmacological agent such as previously described gambogic acid which simultaneously induces different mode of cell death. In general, autophagic cell death is defined by overwhelming degradation of components with essential function in cells. Morphologically, it involves massive generation of vacuoles in cytoplasm and with no chromatin condensation in the process (Shen et al., 2012). On the other hand, stress-induced permeabilization of lysosomal membrane could be a possible autophagy-related mechanism that initiates cell death program. Lysosomal hydrolytic proteases, for instance, cathepsins, are highly hazardous to cytoplasmic components and its release has been showed to mediate apoptosis triggered by oxidative stress (Kagedal et al., 2001). Furthermore, lysosome is an acidic compartment with abundant thiols that reserve iron and copper in their reduced state. Elevated oxidative stress which causes lysosomal membrane permeabilization and ensuing release of the content might contribute to the extensive oxidative damage (Kurz et al., 2010).

Protective Role of Autophagy in Oxidative Stress–Induced Cell Death

Lately with advanced genetic tools to analyze the autophagic machinery, increasing evidence suggested that autophagy functions as a futile fight back of dying cells to survive from fatal stress rather than a form of programmed cell death (Shen et al., 2012). Increased expression of Beclin-1 and suppressed mTOR signaling were observed in human glioma cells with H_2O_2 treatment and inhibition of autophagy stimulates apoptosis in treated cells (Zhang et al., 2009). ROS formation is important for chemotherapeutic agent cisplatin to induce cell death in cancer cells but autophagy was found to reduce cytotoxicity effect of cisplatin (Kaminskyy et al., 2012). Consistently these studies demonstrated prosurvival effect of autophagy against oxidative stress–induced cell death in tumors. There are many mechanisms proposed to give insight for the prosurvival role of autophagy. In this section, we would focus on autophagy-mediated transcriptional regulation of antioxidant defenses in cells.

In maintenance of a homeostatic state, cells need to response adaptively to various stress. The transcription factor, NFE2-related factor 2 (Nrf2), plays an important role in cells to integrate cellular stress signals and to respond by regulating various gene expression. In response to oxidative stress, Nrf2 regulates the cis-acting promoter sequences known as antioxidant response elements and activates the transcription of a range of antioxidant

genes. Under homeostatic condition, Nrf2 is inactive by binding to its negative regulator kelch-like ECH-associated protein 1 (Keap1) in cytoplasm. In complex with Keap1–Cul3, Nrf2 is targeted for degradation by UPS. It is worth noting that the Nrf2–Keap1 system is part of the thiol protein network whose activity is controlled by modifications of cysteine residues in Keap1. Once oxidative agent modifies Keap1, Nrf2 which is released from the complex will translocate into nucleus for regulation of antioxidant gene expression (Brigelius-Flohe and Flohe, 2011).

p62/SQSTM1 (sequestosome 1), which functions as cargo receptor for autophagy-mediated degradation of ubiquitinated proteins, is one of the target genes of Nrf2. At the same time, p62 mediates autophagic degradation of Keap1-activating Nrf2 and thus forms a positive feedback loop in response to oxidative stress (Jain et al., 2010). Mitoquinone (MitoQ), a mitochondrially-targeted redox-active ubiquinone conjugate, was found to effectively kill breast cancer cells via excessive ROS induction in treated cells. Inhibition of Atg7-dependent autophagy was found to enhance the killing effect of MitoQ (Gonzalez et al., 2014). Together these findings imply the fate of cancer cells treated with redox-active therapeutic agents is mainly determined by the interplay between oxidative stress and autophagy-mediated stress response.

Phytoagents in Cancer Treatment

Plants with resourceful bioactive compounds have a long history of use in the treatment of cancer (Cragg et al., 2009). Recently, various phytocompounds, such as genistein, curcumin, (-)-epigallocatechin-3-gallate, and resveratrol, were found to exert antitumor activities by regulating different cell signaling pathways. Emerging evidence suggests that these compounds may enhance antitumor activity through synergistic action or by compensating the inverse properties of current cancer therapies when used in combination (Sarkar and Li, 2006). Meanwhile, many have argued that barriers of current cancer treatments can be avoided by focusing on cancer prevention. In the process of identifying preventive agents, plant-derived phytochemicals which are thought to be safe for human use and proved to possess chemopreventive or chemotherapeutic properties have attracted a great deal of interest (Lee et al., 2011a). Plant-derived bioactive compounds, which are also termed plant secondary metabolites, play important roles in the adaptation of plants to their environment. Large portion of these compounds protect plants from oxygen-induced damage. Therefore, phytochemicals such as flavonoids are originally identified as important antioxidants (Bourgaud et al., 2001; Roleira et al., 2015). Intriguingly growing evidence demonstrates a number of phytochemicals induce ROS only in cancer cells, not in normal cells (Trachootham et al., 2006; Antosiewicz et al., 2008). On the other hand, anticancer phytochemicals were recently reported to initiate autophagy in cancer cells (Wang and Feng, 2015). In the following section, the complicated interplay between ROS induction and autophagy-mediated stress response induced by pro-oxidative phytoagents in cancer cells will be discussed.

Pro-oxidative Phytoagents Induce Autophagy and Cell Death in Cancers

In a study investigating anticancer property of soy isoflavone genistein, selective cytotoxicity was observed in treated MCF-7 breast cancer cells but not in normal fibroblast cells. Increased expression of antioxidant enzymes such as MnSOD, CuZnSOD, and TrxR was

observed in cancer cells with genistein treatment. This result suggests anticancer effect of genistein involves ROS induction that leads to cell death caused by both apoptosis and autophagy in MCF-7 cells (Prietsch et al., 2014). Curcumin, a polyphenolic phytochemical extracted from turmeric *Curcuma longa*, is a pleiotropic redox regulator involved in multiple cellular activities as a pro/antioxidant as recently reviewed (Lopez-Lazaro, 2008). Selective toxicity of curcumin in tumor has been widely investigated. A number of studies indicate curcumin triggers ROS-mediated apoptotic cell death in cancer cells, but not in normal cells (Syng-Ai et al., 2004). In HCT116 human colon cancer cells, curcumin treatment which resulted in ROS induction was found to regulate autophagosome formation and autolysosomal degradation that lead to autophagic cell death. In addition, stress regulator Jun N-terminal kinase (JNK) was activated which may enhance killing effect of curcumin in colon cancer cells (Lee et al., 2011b). In other human colon cancer cell lines, a grape polyphenol compound resveratrol induced ROS which caused autophagy and apoptosis in treated HT-29 and COLO 201 cells. Scavenging of ROS inhibited autophagy and abolished caspase activation while suppression of apoptosis increased autophagic activity as characterized by elevated LC3-II levels in these colon cancer cells. Apparently ROS-triggered autophagy significantly modulates proapoptotic effect of resveratrol (Miki et al., 2012). Saxifragifolin D (SD) is a phytocompound isolated from *Androsace umbellate*. SD induced both apoptosis and autophagy in MCF-7 and MDA-MB-231 breast cancer cells. Molecular studies suggest ROS-mediated ER stress is the common pathway regulating apoptosis and autophagy in SD-treated cancer cells (Shi et al., 2013). Collectively these pro-oxidative phytocompounds showed remarkable inhibitory effects on tumor growth through modulation of apoptotic and autophagic cell death simultaneously. We hypothesize that phytocompound-induced oxidative stress may cause membrane permeabilization of autophagolysosome in late stage of autophagic process. The ensuing release of hydrolytic enzymes and stress signal triggered programmed cell death such as apoptosis synergistically to enhance anticancer effects of these phytoagents (Table 17.1).

Autophagy Attenuates Anticancer Effect of Pro-oxidative Phytoagents

In some cases, pro-oxidant-induced autophagy does not inhibit tumor growth. Quercetin, a common dietary flavonoid, was tested with anticancer properties against many cancers. Early studies suggested quercetin acted as antioxidant but lately quercetin was found to induce ROS and subsequently promote apoptosis and cell cycle arrest in cervical carcinoma cells (Bishayee et al., 2013). In another study, anticancer effect of quercetin was examined using U373MG malignant glioma cells expressing mutant p53. The cell line was resistant to quercetin at 24 h. However when the treatment prolonged to 48 h and 72 h, the compound effectively inhibited tumor cell growth. Inhibition of autophagy by CQ strongly enhanced killing effect of quercetin. Together the observations infer the resistant response showed in early treatment time point may be due to quercetin-induced protective autophagy in treated cells. However, simultaneous induction of stress-induced pathway involves JNK by quercetin eventually led U373MG cells to death (Kim et al., 2013). Some studies suggested targeting altered redox status in head and neck squamous cell carcinoma (HNSCC) can be a unique strategy for its treatment. Therefore, the effectiveness of grape seed extract (GSE) to target aberrant redox regulation in HNSCC was investigated. The result shows that GSE was found to target mitochondrial electron transport chain complex III and to induce oxidative and metabolic stress

TABLE 17.1 Pro-oxidative Phytoagents Induce Autophagy in Cancer Cells

Phytoagent	Cancer Model	Induced Autophagy	Anticancer Activities	Reference
Genistein	MCF-7 breast cancer cells	• Increases expression of antioxidant enzymes such as MnSOD, CuZnSOD and TrxR	• Induces ROS that leads to cell death caused by both apoptosis and autophagy	Prietsch et al. (2014)
Curcumin	HCT116 human colon cancer cells	• Regulates autophagosome formation and autolysosomal degradation	• Activates stress regulator Jun N-terminal kinase (JNK) • Induces autophagic cell death to enhance killing effect of curcumin	Lee et al. (2011b)
	MUH7 human hepatoma cells	• Activates Nrf2 and MAP kinases	• Generates ROS • At low dose, induces HO-1, protects the cells from injury • At high dose, causes cell death via permeability transition pore in mitochondria	McNally et al. (2007)
Resveratrol	HT-29 and COLO 201 colon cancer cells	• Induces ROS-dependent autophagy • Elevates LC3-II levels	• Induces apoptosis in treated cancer cells	Miki et al. (2012)
	K562 chronic myolegenous leukemia (CML)	• Triggers autophagy via JNK dependent accumulation of p62 • Stimulates AMPK resulting in inhibition of mTOR pathway	• Induces autophagy via JNK and AMPK pathways • Mediates autophagic cell death by caspase-independent mechanism	Puissant et al. (2010)
Saxifragifolin D	MCF7 and MDA-MB-231 breast cancer cells	• Increases autophagic vacuoles in cells • Upregulates LC3-II, Beclin1, and Vps34	• Induces interplay between apoptosis and autophagy through ROS-mediated endoplasmic reticulum stress	Shi et al. (2013)
Quercetin	U373MG malignant glioma cells	• Induces large numbers of autophagolysosome vacuoles in cells • Triggers protective autophagy	• Activates JNK and increase expression of p53 • Promotes cell apoptosis via inhibition of autophagy by chloroquine	Kim et al. (2013)
Grape seed extract	Head and neck squamous cell carcinoma	• Activation of autophagy via suppression of Akt/mTOR/4E-BP1/S6K pathway	• Targets mitochondrial electron transport chain complex III • Induces oxidative and metabolic stress and causes apoptotic death	Shrotriya et al. (2014)

leading to apoptotic death in cancer cells. In these treated cells, autophagy was activated via suppression of Akt/mTOR/4E-BP1/S6K pathway and inhibition of autophagic activities significantly augmented the apoptosis (Shrotriya et al., 2014). Apparently both quercetin and grape seed extract stimulate protective autophagy in cancer cells which lessen their killing effect. In previous section, we discussed the synergistic effect of autophagy activation by resveratrol and curcumin in enhanced cancer cell death. However, recent therapeutic approach

which combined several therapeutic agents discovered that inhibition of autophagy could remarkably improve outcome of cancer therapy involving these pro-oxidative phytoagents. CQ is a commonly used autophagy inhibitor that inhibits fusion between autophagosomes and lysosomes, resulting in accumulation of autophagosome in cells. Addition of CQ sensitizes melanoma cells in treatment with ursolic acid and resveratrol (Junco et al., 2015). CQ can also enhance effectiveness of curcumin/temozolomide combination treatment of glioblastoma (Zanotto-Filho et al., 2015). In general, metabolic stress generated by therapeutic agents in the process of treatment may stimulate protective autophagy as a prosurvival mechanism in response to the metabolic stress triggered in treated cancer cells.

FINAL REMARKS

Continued rigorous research to identify molecular targets of phytoagents is important to characterize molecular mechanism of their action in tumors and to provide scientific proof for their use in cancer therapy. As increased tumor resistance to therapy is a direct effect of defects in apoptosis, it is encouraging that several pro-oxidative phytoagents are able to induce both autophagic and apoptotic cell death, thereby maximizing their killing effect in cancers. However in some cases, phytoagents-induced autophagy can also be cytoprotective and cause insensitivity of cancer cells in treatment. Therefore, investigation of the mechanisms is of priority to elaborate the role of phytoagents-related autophagy in deciding cell death versus survival cancer cells. It has been known that the anticancer properties of phytoagents involve modulation of multiple pathways that regulate different stages of carcinogenesis. In this review, we notice most of the current studies investigating regulation of phytoagents on autophagy is mainly based on their effect on formation of autophagosome characterized by LC3 protein expression. However, pleitotropic effects of phytoagents probably regulate autophagy at different levels through various pathways. Therefore, study on each key step of autophagic activity at different stages of cancer in treatment of phytoagents is required to characterize effect of phytoagent on autophagy. Comprehensive information obtained therefrom is important to ensure synergistic effect of phytoagent in cancer therapy. In addition, careful dose-response and stage-dependent studies that compare induction of oxidative stress and autophagy by phytoagents are essential to understand when and to what extent these compounds can be used in combination with other drugs to provide novel therapeutic strategies to overcome the problem of resistance in cancer therapy.

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References

Abdel Malek, M.A., Jagannathan, S., Malek, E., et al., 2015. Molecular chaperone GRP78 enhances aggresome delivery to autophagosomes to promote drug resistance in multiple myeloma. Oncotarget 6, 3098–3110.

Akar, U., Chaves-Reyez, A., Barria, M., et al., 2008. Silencing of Bcl-2 expression by small interfering RNA induces autophagic cell death in MCF-7 breast cancer cells. Autophagy 4, 669–679.

Alexander, A., Cai, S.L., Kim, J., et al., 2010. ATM signals to TSC2 in the cytoplasm to regulate mTORC1 in response to ROS. Proc. Natl. Acad. Sci. U. S. A. 107, 4153–4158.
Amaravadi, R.K., Lippincott-Schwartz, J., Yin, X.M., et al., 2011. Principles and current strategies for targeting autophagy for cancer treatment. Clin. Cancer Res. 17, 654–666.
Antosiewicz, J., Ziolkowski, W., Kar, S., et al., 2008. Role of reactive oxygen intermediates in cellular responses to dietary cancer chemopreventive agents. Planta Med. 74, 1570–1579.
Barnard, R.A., Wittenburg, L.A., Amaravadi, R.K., et al., 2014. Phase I clinical trial and pharmacodynamic evaluation of combination hydroxychloroquine and doxorubicin treatment in pet dogs treated for spontaneously occurring lymphoma. Autophagy 10, 1415–1425.
Bishayee, K., Ghosh, S., Mukherjee, A., et al., 2013. Quercetin induces cytochrome-c release and ROS accumulation to promote apoptosis and arrest the cell cycle in G2/M, in cervical carcinoma: signal cascade and drug-DNA interaction. Cell Prolif. 46, 153–163.
Bourgaud, F., Gravot, A., Milesi, S., et al., 2001. Production of plant secondary metabolites: a historical perspective. Plant Sci. 161, 839–851.
Brigelius-Flohe, R., Flohe, L., 2011. Basic principles and emerging concepts in the redox control of transcription factors. Antioxid. Redox Signal. 15, 2335–2381.
Bruning, A., Juckstock, J., 2015. Misfolded proteins: from little villains to little helpers in the fight against cancer. Front. Oncol. 5 Article 47.
Carew, J.S., Medina, E.C., Esquivel II, J.A., et al., 2010. Autophagy inhibition enhances vorinostat-induced apoptosis via ubiquitinated protein accumulation. J. Cell. Mol. Med. 14, 2448–2459.
Chaabane, W., User, S.D., El-Gazzah, M., et al., 2013. Autophagy, apoptosis, mitoptosis and necrosis: interdependence between those pathways and effects on cancer. Arch. Immunol. Ther. Exp. (Warsz) 61, 43–58.
Chen, N., Karantza-Wadsworth, V., 2009. Role and regulation of autophagy in cancer. Biochim. Biophys. Acta 1793, 1516–1523.
Cragg, G.M., Grothaus, P.G., Newman, D.J., 2009. Impact of natural products on developing new anti-cancer agents. Chem. Rev. 109, 3012–3043.
David, E., Kaufman, J.L., Flowers, C.R., et al., 2010. Tipifarnib sensitizes cells to proteasome inhibition by blocking degradation of bortezomib-induced aggresomes. Blood 116, 5285–5288.
Degenhardt, K., Mathew, R., Beaudoin, B., et al., 2006. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. Cancer Cell 10, 51–64.
Ding, W.X., Yin, X.M., 2008. Sorting, recognition and activation of the misfolded protein degradation pathways through macroautophagy and the proteasome. Autophagy 4, 141–150.
Duffy, A., Le, J., Sausville, E., et al., 2015. Autophagy modulation: a target for cancer treatment development. Cancer Chemother. Pharmacol. 75, 439–447.
Ertmer, A., Huber, V., Gilch, S., et al., 2007. The anticancer drug imatinib induces cellular autophagy. Leukemia 21, 936–942.
Fang, J., Seki, T., Maeda, H., 2009. Therapeutic strategies by modulating oxygen stress in cancer and inflammation. Adv. Drug Deliv. Rev. 61, 290–302.
Fang, Y., Vilella-Bach, M., Bachmann, R., et al., 2001. Phosphatidic acid-mediated mitogenic activation of mTOR signaling. Science 294, 1942–1945.
Filomeni, G., De Zio, D., Cecconi, F., 2015. Oxidative stress and autophagy: the clash between damage and metabolic needs. Cell Death Differ. 22, 377–388.
Glick, D., Barth, S., Macleod, K.F., 2010. Autophagy: cellular and molecular mechanisms. J. Pathol. 221, 3–12.
Goldberg, S.B., Supko, J.G., Neal, J.W., et al., 2012. A phase I study of erlotinib and hydroxychloroquine in advanced non-small-cell lung cancer. J. Thorac. Oncol. 7, 1602–1608.
Gonzalez, Y., Aryal, B., Chehab, L., et al., 2014. Atg7- and Keap1-dependent autophagy protects breast cancer cell lines against mitoquinone-induced oxidative stress. Oncotarget 5, 1526–1537.
Gutierrez, M.G., Master, S.S., Singh, S.B., et al., 2004. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. Cell 119, 753–766.
Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. Cell 144, 646–674.
Hao, R., Nanduri, P., Rao, Y., et al., 2013. Proteasomes activate aggresome disassembly and clearance by producing unanchored ubiquitin chains. Mol. Cell 51, 819–828.
Harding, H.P., Calfon, M., Urano, F., et al., 2002. Transcriptional and translational control in the mammalian unfolded protein response. Annu. Rev. Cell Dev. Biol. 18, 575–599.

Hasima, N., Ozpolat, B., 2014. Regulation of autophagy by polyphenolic compounds as a potential therapeutic strategy for cancer. Cell Death Dis. 5, e1509.
Ishaq, M., Khan, M.A., Sharma, K., et al., 2014. Gambogic acid induced oxidative stress dependent caspase activation regulates both apoptosis and autophagy by targeting various key molecules (NF-kappaB, Beclin-1, p62 and NBR1) in human bladder cancer cells. Biochim. Biophys. Acta 1840, 3374–3384.
Jain, A., Lamark, T., Sjottem, E., et al., 2010. p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription. J. Biol. Chem. 285, 22576–22591.
Jiang, S., Park, D.W., Gao, Y., et al., 2015. Participation of proteasome-ubiquitin protein degradation in autophagy and the activation of AMP-activated protein kinase. Cell Signal. 27, 1186–1197.
Junco, J.J., Mancha-Ramirez, A., Malik, G., et al., 2015. Ursolic acid and resveratrol synergize with chloroquine to reduce melanoma cell viability. Melanoma Res. 25, 103–112.
Kagedal, K., Johansson, U., Ollinger, K., 2001. The lysosomal protease cathepsin D mediates apoptosis induced by oxidative stress. FASEB J. 15, 1592–1594.
Kaminskyy, V.O., Piskunova, T., Zborovskaya, I.B., et al., 2012. Suppression of basal autophagy reduces lung cancer cell proliferation and enhances caspase-dependent and -independent apoptosis by stimulating ROS formation. Autophagy 8, 1032–1044.
Kaufmann, A.M., Krise, J.P., 2007. Lysosomal sequestration of amine-containing drugs: analysis and therapeutic implications. J. Pharm. Sci. 96, 729–746.
Kim, D.H., Sarbassov, D.D., Ali, S.M., et al., 2002. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell 110, 163–175.
Kim, H., Moon, J.Y., Ahn, K.S., 2013. Quercetin induces mitochondrial mediated apoptosis and protective autophagy in human glioblastoma U373MG cells. Oxid. Med. Cell. Longev. 2013 Article 596496.
Komatsu, M., Waguri, S., Ueno, T., et al., 2005. Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. J. Cell Biol. 169, 425–434.
Komatsu, M., Kurokawa, H., Waguri, S., et al., 2010. The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. Nat. Cell Biol. 12, 213–223.
Komatsu, S., Moriya, S., Che, X.F., et al., 2013. Combined treatment with SAHA, bortezomib, and clarithromycin for concomitant targeting of aggresome formation and intracellular proteolytic pathways enhances ER stress-mediated cell death in breast cancer cells. Biochem. Biophys. Res. Commun. 437, 41–47.
Kouroku, Y., Fujita, E., Tanida, I., et al., 2007. ER stress (PERK/eIF2alpha phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation. Cell Death Differ. 14, 230–239.
Kriegenburg, F., Poulsen, E.G., Koch, A., et al., 2011. Redox control of the ubiquitin-proteasome system: from molecular mechanisms to functional significance. Antioxid. Redox Signal. 15, 2265–2299.
Kurz, T., Eaton, J.W., Brunk, U.T., 2010. Redox activity within the lysosomal compartment: implications for aging and apoptosis. Antioxid. Redox Signal. 13, 511–523.
Lee, K.W., Bode, A.M., Dong, Z., 2011a. Molecular targets of phytochemicals for cancer prevention. Nat. Rev. Cancer 11, 211–218.
Lee, W.L., Shyur, L.F., 2012. Deoxyelephantopin impedes mammary adenocarcinoma cell motility by inhibiting calpain-mediated adhesion dynamics and inducing reactive oxygen species and aggresome formation. Free Radic. Biol. Med. 52, 1423–1436.
Lee, W.L., Wen, T.N., Shiau, J.Y., et al., 2009. Differential proteomic profiling identifies novel molecular targets of paclitaxel and phytoagent deoxyelephantopin against mammary adenocarcinoma cells. J. Proteome Res. 9, 237–253.
Lee, Y.J., Kim, N.Y., Suh, Y.A., et al., 2011b. Involvement of ROS in curcumin-induced autophagic cell death. Korean J. Physiol. Pharmacol. 15, 1–7.
Liang, X.H., Jackson, S., Seaman, M., et al., 1999. Induction of autophagy and inhibition of tumorigenesis by Beclin 1. Nature 402, 672–676.
Livesey, K.M., Kang, R., Vernon, P., et al., 2012. p53/HMGB1 complexes regulate autophagy and apoptosis. Cancer Res. 72, 1996–2005.
Lopez-Lazaro, M., 2008. Anticancer and carcinogenic properties of curcumin: considerations for its clinical development as a cancer chemopreventive and chemotherapeutic agent. Mol. Nutr. Food Res. 52 (Suppl. 1), S103–S127.
MacCallum, S.F., Groves, M.J., James, J., et al., 2013. Dysregulation of autophagy in chronic lymphocytic leukemia with the small-molecule Sirtuin inhibitor Tenovin-6. Sci. Rep. 3 Article 1275.

Mahalingam, D., Mita, M., Sarantopoulos, J., et al., 2014. Combined autophagy and HDAC inhibition: a phase I safety, tolerability, pharmacokinetic, and pharmacodynamic analysis of hydroxychloroquine in combination with the HDAC inhibitor vorinostat in patients with advanced solid tumors. Autophagy 10, 1403–1414.

Mcnally, S.J., Harrison, E.M., Ross, J.A., et al., 2007. Curcumin induces heme oxygenase 1 through generation of reactive oxygen species, p38 activation and phosphatase inhibition. Int. J. Mol. Med. 19, 165–172.

Miki, H., Uehara, N., Kimura, A., et al., 2012. Resveratrol induces apoptosis via ROS-triggered autophagy in human colon cancer cells. Int. J. Oncol. 40, 1020–1028.

Ogata, M., Hino, S., Saito, A., et al., 2006. Autophagy is activated for cell survival after endoplasmic reticulum stress. Mol. Cell. Biol. 26, 9220–9231.

Ogawa, M., Yoshimori, T., Suzuki, T., et al., 2005. Escape of intracellular *Shigella* from autophagy. Science 307, 727–731.

Otomo, C., Metlagel, Z., Takaesu, G., et al., 2013. Structure of the human ATG12~ATG5 conjugate required for LC3 lipidation in autophagy. Nat. Struct. Mol. Biol. 20, 59–66.

Pattingre, S., Tassa, A., Qu, X., et al., 2005. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell 122, 927–939.

Prietsch, R.F., Monte, L.G., Da Silva, F.A., et al., 2014. Genistein induces apoptosis and autophagy in human breast MCF-7 cells by modulating the expression of proapoptotic factors and oxidative stress enzymes. Mol. Cell. Biochem. 390, 235–242.

Pua, H.H., Dzhagalov, I., Chuck, M., et al., 2007. A critical role for the autophagy gene Atg5 in T cell survival and proliferation. J. Exp. Med 204, 25–31.

Puissant, A., Robert, G., Fenouille, N., et al., 2010. Resveratrol promotes autophagic cell death in chronic myelogenous leukemia cells via JNK-mediated p62/SQSTM1 expression and AMPK activation. Cancer Res. 70, 1042–1052.

Rangwala, R., Chang, Y.C., Hu, J., et al., 2014a. Combined MTOR and autophagy inhibition: phase I trial of hydroxychloroquine and temsirolimus in patients with advanced solid tumors and melanoma. Autophagy 10, 1391–1402.

Rangwala, R., Leone, R., Chang, Y.C., et al., 2014b. Phase I trial of hydroxychloroquine with dose-intense temozolomide in patients with advanced solid tumors and melanoma. Autophagy 10, 1369–1379.

Rodriguez-Gonzalez, A., Lin, T., Ikeda, A.K., et al., 2008. Role of the aggresome pathway in cancer: targeting histone deacetylase 6-dependent protein degradation. Cancer Res. 68, 2557–2560.

Roleira, F.M., Tavares-Da-Silva, E.J., Varela, C.L., et al., 2015. Plant derived and dietary phenolic antioxidants: anticancer properties. Food Chem. 183, 235–258.

Rosenfeld, M.R., Ye, X., Supko, J.G., et al., 2014. A phase I/II trial of hydroxychloroquine in conjunction with radiation therapy and concurrent and adjuvant temozolomide in patients with newly diagnosed glioblastoma multiforme. Autophagy 10, 1359–1368.

Rosich, L., Xargay-Torrent, S., Lopez-Guerra, M., et al., 2012. Counteracting autophagy overcomes resistance to everolimus in mantle cell lymphoma. Clin. Cancer Res. 18, 5278–5289.

Rubinsztein, D.C., Codogno, P., Levine, B., 2012. Autophagy modulation as a potential therapeutic target for diverse diseases. Nat. Rev. Drug Discov. 11, 709–730.

Sarkar, F.H., Li, Y., 2006. Using chemopreventive agents to enhance the efficacy of cancer therapy. Cancer Res. 66, 3347–3350.

Schumacker, P.T., 2006. Reactive oxygen species in cancer cells: live by the sword, die by the sword. Cancer Cell 10, 175–176.

Shao, Y., Gao, Z., Marks, P.A., et al., 2004. Apoptotic and autophagic cell death induced by histone deacetylase inhibitors. Proc. Natl. Acad. Sci. U.S.A. 101, 18030–18035.

Shen, S., Kepp, O., Kroemer, G., 2012. The end of autophagic cell death? Autophagy 8, 1–3.

Shi, J.M., Bai, L.L., Zhang, D.M., et al., 2013. Saxifragifolin D induces the interplay between apoptosis and autophagy in breast cancer cells through ROS-dependent endoplasmic reticulum stress. Biochem. Pharmacol. 85, 913–926.

Shrotriya, S., Deep, G., Lopert, P., et al., 2014. Grape seed extract targets mitochondrial electron transport chain complex III and induces oxidative and metabolic stress leading to cytoprotective autophagy and apoptotic death in human head and neck cancer cells. Mol. Carcinog. [Epub ahead of print].

Simizu, S., Takada, M., Umezawa, K., et al., 1998. Requirement of caspase-3(-like) protease-mediated hydrogen peroxide production for apoptosis induced by various anticancer drugs. J. Biol. Chem. 273, 26900–26907.

Sotelo, J., Briceno, E., Lopez-Gonzalez, M.A., 2006. Adding chloroquine to conventional treatment for glioblastoma multiforme: a randomized, double-blind, placebo-controlled trial. Ann. Intern. Med. 144, 337–343.
Syng-Ai, C., Kumari, A.L., Khar, A., 2004. Effect of curcumin on normal and tumor cells: role of glutathione and bcl-2. Mol. Cancer Ther. 3, 1101–1108.
Trachootham, D., Zhou, Y., Zhang, H., et al., 2006. Selective killing of oncogenically transformed cells through a ROS-mediated mechanism by beta-phenylethyl isothiocyanate. Cancer Cell 10, 241–252.
Tsujimoto, Y., Shimizu, S., 2005. Another way to die: autophagic programmed cell death. Cell Death Differ. 12, 1528–1534.
Vogl, D.T., Stadtmauer, E.A., Tan, K.S., et al., 2014. Combined autophagy and proteasome inhibition: a phase 1 trial of hydroxychloroquine and bortezomib in patients with relapsed/refractory myeloma. Autophagy 10, 1380–1390.
Wang, N., Feng, Y., 2015. Elaborating the role of natural products-induced autophagy in cancer treatment: achievements and artifacts in the state of the art. Biomed. Res. Int. 2015 Article 934207.
Xie, X., White, E.P., Mehnert, J.M., 2013. Coordinate autophagy and mTOR pathway inhibition enhances cell death in melanoma. PLoS One 8, e55096.
Yang, Z.J., Chee, C.E., Huang, S., et al., 2011. The role of autophagy in cancer: therapeutic implications. Mol. Cancer Ther. 10, 1533–1541.
Yogalingam, G., Pendergast, A.M., 2008. Abl kinases regulate autophagy by promoting the trafficking and function of lysosomal components. J. Biol. Chem. 283, 35941–35953.
Yue, Z., Jin, S., Yang, C., et al., 2003. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. Proc. Natl. Acad. Sci. U.S.A. 100, 15077–15082.
Zanotto-Filho, A., Braganhol, E., Klafke, K., et al., 2015. Autophagy inhibition improves the efficacy of curcumin/temozolomide combination therapy in glioblastomas. Cancer Lett. 358, 220–231.
Zhang, H., Kong, X., Kang, J., et al., 2009. Oxidative stress induces parallel autophagy and mitochondria dysfunction in human glioma U251 cells. Toxicol Sci. 110, 376–388.
Zhu, K., Dunner Jr., K., Mcconkey, D.J., 2010. Proteasome inhibitors activate autophagy as a cytoprotective response in human prostate cancer cells. Oncogene 29, 451–462.

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	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
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 autophagosome
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 macroautophagy
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
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 is 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	mitafusin 1 is required 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	phosphatidyl ethanolamine

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
Syt1	synaptotagmin 1
T1DM	type 1 diabetes mellitus
TAKA	transport of Atg9 after knocking-out Atg1
TASCC	TOR-autophagy spatial coupling compartment
TCN	trans-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.

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