CHAPTER 1

Introduction to Autophagy: Cancer, Other Pathologies, Inflammation, Immunity, Infection, and Aging, Volume 5

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OUTLINE

Introduction 2
Specific Functions of Autophagy (A Summary) 4
Autophagy in Normal Mammalian Cells 4
Endoplasmic Reticulum Stress and Autophagy 5
Major Types of Autophagies
  Macroautophagy (Autophagy) 6
  Microautophagy 7
  Chaperone-Mediated Autophagy 7
Autophagosome Formation 8
Autophagic Lysosome Reformation 9
Autophagic Proteins 10

Protein Degradation Systems 11
  Beclin 1 11
  Non-Autophagic Functions of Autophagy-Related Proteins 12
  Microtubule-Associated Protein Light Chain 3 13

Monitoring Autophagy 13
Reactive Oxygen Species (ROS) 14
Mammalian Target of Rapamycin (mTOR) 14
Role of Autophagy in Tumorigenesis and Cancer 15
Role of Autophagy in Immunity 17
Autophagy and Senescence 19
INTRODUCTION

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

Regarding the role of a healthy diet, a caloric restriction induces autophagy that counteracts the development of age-related diseases and aging itself. On the other hand, autophagy is inhibited by high glucose and insulin-induced P13K signaling via Akt and mTOR. Based on its fundamental roles in these and other disease processes’ prevention and therapy, autophagy has emerged as a potential target for disease.
Unfortunately, inevitable death rules our lives, and a group of abnormal cells plays a part in it. Safe disposal of cellular debris is crucial to keep us alive and healthy. Our body uses autophagy and apoptosis as clearing mechanisms to eliminate malfunctioning, aged, damaged, excessive, and/or pathogen-infected cell debris that might otherwise be harmful/autoimmunogenic. However, if such a clearing process becomes uncontrollable, it can instead be deleterious. For example, deficits in protein clearance in brain cells because of dysfunctional autophagy may lead to dementia. Autophagy can also promote cell death through excessive self-digestion and degradation of essential cellular constituents.

Humans and other mammals with long lifespans unfortunately have to face the problem of the accumulation of somatic mutations over time. Although most of the mutations are benign and only some lead to disease, there are too many of them. Cancer is one of these major diseases, and is caused by a combination of somatic genetic alterations in a single cell, followed by uncontrolled cell growth and proliferation. Even a single germline deletion of or mutation in a tumor suppressor gene (e.g., \textit{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 (\textit{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 \textit{autophagy} (\textit{auto phagin}, from the Greek meaning self-eating) refers to a phenomenon in which cytoplasmic components are delivered to the lysosomes for bulk or selective degradation under the lysosomes’ distinct intracellular and extracellular milieu. This term was first coined by de Duve over 46 years ago (Deter and de Duve, 1967), based on the observed degradation of mitochondria and other intracellular structures within lysosomes of rat liver perfused with the pancreatic hormone glucagon.

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

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

Autophagy plays a constitutive and basally active role in the quality control of proteins and organelles, and is associated with either cell survival or cell death. Stress-responsive autophagy can enable adaptation and promote cell survival, whereas in certain models, autophagy has also been associated with cell death, representing either a failed attempt at survival or a mechanism that supports cell and tissue degradation. Autophagy prevents the accumulation of random molecular damage in long-lived structures, particularly mitochondria, and more generally provides a means to reallocate cellular resources from one biochemical pathway to another. Consequently, it is upregulated in conditions where a cell is
responding to stress signals, such as starvation, oxidative stress, and exercise-induced adaptation. The balance between protein and lipid biosynthesis, and their eventual degradation and resynthesis, is one critical component of cellular health.

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

**SPECIFIC FUNCTIONS OF AUTOPHAGY (A SUMMARY)**

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

**AUTOPHAGY IN NORMAL MAMMALIAN CELLS**

Although autophagy mediates cell adaptation to a range of stress conditions, including starvation, this stress is not a problem that a normal cell of a multicellular organism would face on a regular basis. The basal level of autophagy (the so-called basal or quality control autophagy) is found in most cells, and is required for the normal clearance of potentially deleterious protein aggregates that can cause cellular dysfunction. Thus, mammalian autophagy is primarily required for intracellular cleaning of misfolded proteins.
and damaged/old organelles. In the absence of such cleaning, neoplastic transformation is likely.

As alluded to above, starvation is uncommon in mammalian cells under normal nutritional conditions. Therefore, it is important to know the mechanism responsible for regulating autophagy under normal nutritional conditions. In mammalian cells, mTOR kinase, the target of rapamycin, mediates a major inhibitory signal that represses autophagy under nutrient-rich conditions. Calpain 1 keeps autophagy under tight control by downregulating the levels of Atg12–Atg5 conjugate. Atg5 and Atg12–Atg5 conjugate are key signaling molecules for increasing the levels of autophagy (Xia et al., 2010). It is also known that intracellular Ca\(^{2+}\) regulates autophagy. Inhibition of Ca\(^{2+}\) influx results in the induction of autophagy.

Reduction in intracellular Ca\(^{2+}\) prevents the cleavage of Atg5, which in turn increases the levels of full-length Atg5 and Atg12–Atg5 conjugate. The Atg12–Atg5 signaling molecule is regulated by calpain 1 in controlling the levels of autophagy in mammalian cells under nutrient-rich conditions. It is known that inhibition of calpains induces autophagy, and reduces the accumulation of misfolded proteins. It is further known that increased levels of LC3-II in fluspirilene-treated cells promote autophagy by increasing the levels of Atg5 and Atg12–Atg5 conjugate; fluspirilene is one of the autophagy inducers. Although autophagy is maintained at very low levels in normal mammalian cells, it can be rapidly induced within minutes upon starvation or invasion by intracellular pathogens.

ENDOPLASMIC RETICULUM STRESS AND AUTOPHAGY

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 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.

ER stress can be caused by physiological or pathological processes that disturb protein folding in the ER. 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).

The initial and rapid response of cells to the ER stress is the activation of a set of pro-survival signaling pathways called the unfolded protein response (UPR) (Doyle et al., 2011). UPR regulates the 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). Bernales et al. 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., 2006b).

UPR also blocks protein synthesis and activates mechanisms that prepare the cell to cope with the aggregated unfolded proteins. One such mechanism involves the enhancement of the protein folding capacity of the ER by increasing the expression of ER chaperone proteins and upregulating 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 via 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, and 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).
1. INTRODUCTION TO AUTOPHAGY

CMA serves to balance dysregulated energy, and is maximally activated by nutrient/metabolic and oxidative/nitrostatic 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 cytosolic 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 it, along with its ribosomes, is involved in protein synthesis. The presence of many Atg proteins near the ER also suggests that ER plays an important role as a membrane source for autophagosome formation. The formation of isolation membrane is initiated by class III phosphatidylinositol 3-kinase (PI3KC)/Beclin 1-containing complexes. Elongation of the isolation membrane involves two ubiquitin-like conjugation systems. In one of them, Atg12 associates with Atg5 to form Atg12–Atg5–Atg16L1 molecular complexes that bind the outer membrane of the isolation membrane. In the second, lipidated microtubule-associated light chain 3 (LC3) is conjugated to phosphatidylethanolamine to generate a lipidated LC3-II form, which is integrated in both the outer and inner membranes of the autophagosome (Fujita et al., 2008). Recently, it was reported that human Atg2 homologues Atg2A and AtgB are also essential for autophagosome formation, presumably at a late stage (Velikkakath et al., 2012).

Autophagosome membrane formation requires autophagy-related proteins (Atgs) along with the insertion of lipidated microtubule-associated light chain 3 (LC3) or gamma-aminobutyric acid A receptor-associated protein (GABARAP) subfamily members. Various components in the autophagosomal compartment can be recognized by the presence of specific autophagy molecules. Atg16L1 and Atg5 are mainly present in the phagophore, while LC3 labels isolation membranes, matured autophagosomes, and autolysosomes (Gao et al., 2010). This evidence suggests that different Atg molecules participate in autophagosome biogenesis at various stages. Autophagosome substrate selectivity can be conferred by interactions between LC3 and specific cargo receptors, including sequestosome-1 (SQSTM1 #p62) and a neighbor of BRCA1 (NBR1). During this process of autophagy, both lipidated LC3 (LC3-II) and the cargo receptors are degraded (Hocking et al., 2012).

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

**AUTOPHAGIC LYSOSOME REFORMATION**

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

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

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

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

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

**AUTOPHAGIC PROTEINS**

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

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

Autophagy was first genetically defined in yeast, where 31 genes, referred to as autophagy-related genes (ATGs), were identified as being directly involved in the execution of autophagy (Mizushima, 2007; Xie and Klionsky, 2007). At least 16 members of this gene family have been identified in humans. The role of a large number of these genes has been deciphered. Our understanding of the molecular regulation of 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 multispansing 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.

MONITORING AUTOPHAGY

A number of methods are available to monitor autophagy; such monitoring can be accomplished by using electron microscopy, biochemical protocols, and detection of relevant protein modifications through SDS-PAGE and western blotting. Autophagy can be monitored by detecting autophagosomal proteins such as LC3. LC3 is a specific marker protein of autophagic structure in mammalian cultured cells. The appearance of this protein-positive puncta is indicative of the induction of autophagy. One such method consists of monitoring autophagy by detecting LC3 conversion from LC3-I to LC3-II by immunoblot analysis because the amount of LC3-II is clearly correlated with the number of autophagosomes. Endogenous LC3 is detected as two bands following SDS-PAGE and immunoblotting: one represents cytosolic LC3-I and the other, LC3-II that is conjugated with phosphatidylethanolamine, which is present on isolation membranes and autophagosomes but much less so on autolysosomes (Mizushima and Yoshimori, 2007). According to Kadowaki and Karim (2009), the LC3-I to LC3-II ratio in the cytosol (cytosolic LC3 ratio), but not in the homogenate, is an easy quantitative method for monitoring the regulation of autophagy. Alternatively, comparison of LC3-II levels between different conditions is a useful method for monitoring autophagy.
Another approach is use of the fluorescent protein GFP-LC3, which is a simple and specific marker. To analyze autophagy in whole animals, GFP-LC3 transgenic mice have been generated (Mizushima and Kuma, 2008). However, the GFP-LC3 method does not provide a convenient measure for assessing autophagic flux. Therefore, another alternative method, tandem fluorescent-tagged LC# (tfLC#) can be used to monitor autophagic flux (Kimura et al., 2009).

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

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

**REACTIVE OXYGEN SPECIES (ROS)**

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

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

**MAMMALIAN TARGET OF RAPAMYCIN (mTOR)**

The mammalian target of rapamycin (mTOR), also known as the mechanistic target of rapamycin or FK506-binding protein 12-rapamycin-associated protein 1 (FRAP1), is an
-289-kDa protein originally discovered and cloned from *Saccharomyces cerevisiae* that shares sequence homologues with the phosphoinositide 3-kinase (PI3-kinase) family, which is the key element in response to growth factors. mTOR represents a serine threonine protein kinase that is present in all eukaryotic organisms (Wullschleger *et al.*, 2006). mTOR represents the catalytic subunit of two distinct complexes, mTORC1 and mTORC2 (Zoncu *et al.*, 2011). mTORC1 controls cell growth by maintaining a balance between anabolic processes (e.g., macromolecular synthesis and nutrient storage) and catabolic processes (e.g., autophagy and the utilization of energy stores) (Nicoletti *et al.*, 2011). The receptor–mTOR complex positively regulates cell growth, and its inhibition causes a significant decrease in cell size. The raptor part of the mTOR pathway modulates a large number of major processes, 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 sensing. 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 BECN1 gene is deleted in ~40% of prostate cancers, ~50% of breast cancers, and ~75% of ovarian cancers (Liang et al., 1999). In addition, reduced expression of Beclin 1 has been found in other types of cancers, including human colon cancer, brain tumors, hepatocellular carcinoma, and cervical cancer. It can be concluded that a defective autophagic process is clearly linked to cancer development.

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

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

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

**ROLE OF AUTOPHAGY IN IMMUNITY**

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

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

The autophagy pathway is linked to one or more aspects of immunity. Studies have shown that autophagy is regulated by pathways that are critical for the function and differentiation of cells of the immune system, including Toll-like receptors (TLRs). TLRs were the first class of immune receptors identified as regulators in cells of the innate immune system, and play a crucial role in many aspects of the immune response. They are broadly expressed in immune cells, particularly in antigen-presenting cells, and recognize pathogen-associated molecular patterns such as lipopolysaccharides, viral double-stranded RNA, and unmethylated CPG islands (Harashima et al., 2012). Initiation of TLR signaling induces release of inflammatory cytokines, maturation of dendritic cells, and activation of adaptive immunity. Cancer cells also express functional TLRs. TLR4 signaling, for example, promotes escape of human lung cancer cells from the immune system by inducing immune suppressive cytokines and promoting resistance to apoptosis (He et al., 2007). In contrast, TLR3 signaling induces antitumor effects. Akt activation can render cancer cells resistant to antitumor cellular immunity (Hähnel et al., 2008). The implication is that Akt inactivation increases the susceptibility of cancer cells to immune surveillance.

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

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

Cellular senescence is a biological state in which cells have lost the ability 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 cell’s defense mechanisms or to use them for its replication. Autophagy plays a dual role in HIV-1 infection and disease progression. Direct effects of HIV on autophagy include the subversion of autophagy in HIV-infected cells and the induction of hyper-autophagy in bystander CD4+ T cells. HIV proteins modulate autophagy to maximize virus production (Killian, 2012). On the other hand, HIV-1 protein also disrupts autophagy in uninfected cells and thus contributes to CD4+ T cell death and viral pathogenesis.

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

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

**ROLE OF AUTOPHAGY IN INTRACELLULAR BACTERIAL INFECTION**

Post-translation modifications of cell proteins (e.g., ubiquitination) regulate the intracellular traffic of pathogens. Ubiquitination involves the addition of ubiquitin to the lysine
residues of target proteins, resulting in endocytosis and sorting events (Railborg and Stenmark, 2009). Several strategies have been developed by pathogenic bacteria to interfere with the host’s ubiquitination and thus to achieve successful infection. Some types of bacteria act directly on the ubiquitination pathway by mimicking host cell proteins, while others (e.g., Escherichia coli, Shigella flexneri) act indirectly by expressing or interfering with the host ubiquitinating pathway. The other defense by the cell against bacterial infection is through autophagy; this is described below.

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

ROLE OF AUTOPHAGY IN HEART DISEASE

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

It has been reported that autophagy is an adaptive mechanism to protect the heart from hemodynamic stress. In fact, autophagy plays a crucial role in the maintenance of cardiac geometry and contractile function (Nemchenko et al., 2011). Cardiac-specific loss of autophagy causes cardiomyopathy. Impaired autophagy has been found in a number of heart diseases, including ischemia/reperfusion injury. Excessive and uncontrolled autophagy leads to loss of functional proteins, depletion of essential organic molecules, oxidative stress, loss of ATP, the collapse of cellular catabolic machinery, and, ultimately, the death of cells in the heart. Autophagic elimination of damaged organelles, especially mitochondria, is crucial for proper heart function, whereas exaggerated autophagic activity may foster heart failure. Therefore, a delicate balance of autophagy maintains cardiac homeostasis, whereas an imbalance leads to the progression of heart failure.
A consensus on whether autophagy is cardioprotective or leads to hypertrophy and heart failure is lacking. In any case, autophagy is an important process in the heart. Various studies indicate that autophagy has a dual role in the heart, where it can protect against or contribute to cell death depending on the stimulus. It occurs at low basal levels under normal conditions, and is important for the turnover of organelles. Autophagy is upregulated in the heart in response to stress such as ischemia/reperfusion. Studies of ischemia/reperfusion injury indicate that ROS and mitochondria are critical targets of injury, as opening of the mitochondrial permeability transition pore culminates in cell death. However, Sciarretta et al. (2011) indicate that autophagy is beneficial during ischemia but harmful during reperfusion.

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

Recently, it was reported that autophagy plays a role in the onset and progression of alcoholic cardiopathy (Guo and Ren, 2012). Adenosine monophosphate-activated protein kinase (AMPK) plays a role in autophagic regulation and subsequent changes in cardiac function following an alcoholic challenge. It is known that AMPK promotes autophagy via inhibition of mTORC1 by phosphorylating the mTORC1-associated protein Raptor and tuberous sclerosis complex 2. MicroRNAs (miRNAs) also play a role in cardiomyopathy and heart failure. These endogenous small molecules regulate their target gene expression by post-transcriptional regulation of messenger RNA. Recently, it was demonstrated that hypertrophic conditions induced the expression of the miR-212/132 family in cardiomyocytes, and both of these molecules regulated cardiac hypertrophy and cardiomyocyte autophagy (Ucar et al., 2012). Cardiac hypertrophy and heart failure in mice can be rescued by using a pharmacological inhibitor of miR-132.

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

**ROLE OF AUTOPHAGY IN NEURODEGENERATIVE DISEASES**

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

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

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

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

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

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

Amyotrophic lateral sclerosis (ALS) is the fourth most common neurodegenerative disease. It is characterized by progressive loss of upper and motor neurons. The following genes and proteins have been reported to be involved in familial ALS: superoxide dismutase 1, als2, TAR DNA binding protein of 43kDa, 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 substantia 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. Uregulation of autophagy has the potential to be a therapeutic strategy for disorders. This genetic method for autophagy upregulation is mTOR-independent. The development of genetic-based therapeutic strategies aimed at stimulating the autophagic clearance of aggregated proteins can be used both in the treatment of neurodegenerative diseases and in lifespan extension (Zhang et al., 2010). Several studies have identified histone deacetylose (HDAC) inhibitors (4b) as candidate drugs for the treatment of neurological diseases, including HD.

CROSS-TALK BETWEEN AUTOPHAGY AND APOPTOSIS

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

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

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

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

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

The concept of the presence of cross-talk between autophagy and apoptosis is reinforced by the indication that common cellular stresses activate various signaling pathways
which regulate both of these cell death programs. ROS induce apoptosis and regulate Atg4, which is essential for autophagy induction. In addition, Atg5 promotes both apoptosis and autophagy induction. In addition to Atg5, several other signal transduction pathways (Bcl2 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 cytotoxicin-induced autophagy and apoptosis; this toxin induces mitochondrial damage leading to apoptosis. In these cells, the toxin triggers formation of autophagosomes, followed by autolysosome formation. Recently it was reported that death-associated protein kinase (DAPK) induces autophagy in colon cancer cells in
response to treatment with histone deacetylase inhibitor (HDACi), while in autophagy-deficient cells DAPK plays an essential role in committing cells to HDACi-induced apoptosis (Gandesiri et al., 2012).

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

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

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

**AUTOPHAGY AND UBIQUITINATION**

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

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

The ubiquitin proteasome system (UPS) removes non-functional, damaged, and misfolded proteins from the cell. When the capacity of the proteasome is impaired and/or when the amounts of misfolded proteins exceed the capacity of proteasome, they accumulate in the aggresome, the mechanism of which is explained below. Aggresomes are localized in the proximity of the microtubule-organizing center. Microtubule-associated histone deacetylase 6 (HDAC6) mediates this process. Through its ubiquitin-binding BUZ finger domain, HDAC6 binds to and facilitates the transport of polyubiquitinated misfolded proteins along microtubules to the aggresome (Kawaguchi et al., 2003). Aggresome removal is mediated by ubiquitin-binding proteins such as p62/SQSTM1 and NBR1. These adaptor proteins through their ubiquitin-binding protein (UBA) are responsible for the fate of protein degradation either through the UPS or via autophagy (Komatsu and Ichimura, 2010). E3-ubiquitin ligases play a key role in the execution of autophagy (Chin et al., 2010).

Recently, it was reported that in response to proteasome inhibition, the E3-ubiquitin ligase TRIM50 localizes and promotes the recruitment and aggregation of polyubiquitinated proteins to the aggresome (Fusco et al., 2012). Fusco and colleagues showed TRIM50 co-localizes, interacts with, and increases the level of p62, which is a multifunctional adaptor protein involved in various cellular processes including the autophagic clearance of polyubiquitinated protein aggregates. The implication of this information is that in the absence of proteasome activity, TRIM50 fails to drive its substrates to proteasome-mediated degradation and promotes their storage in the aggresome for subsequent removal by p62-mediated autophagy. It is known that the accumulation of polyubiquitinated protein aggregates is associated with neurodegenerative disorders and other protein aggregation diseases. It is also known that p62 is a component of inclusion bodies in neurodegenerative diseases and liver diseases.

AUTOPHAGY AND NECROPTOSIS

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

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

It was recently found that TNF-α administration causes mitochondrial dysfunction and ROS production (Ye et al., 2012). Mitochondrial dysfunction led to necroptosis and autophagy in murine fibrosarcoma L929 cells. Nec-1 represses, whereas pan-caspase inhibitor z-VAD-fmk (z-VAD) increases, RIP1 expression. This increase, in turn, enhances TNF-α induced mitochondrial dysfunction and ROS production. It has also been shown that TNF-α
administration and zVAD induce cytochrome c release from mitochondria, whereas Nec-1 blocks this release (Ye et al., 2012).

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

**MITOCHONDRIAL FUSION AND FISSION**

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

The central components that mediate mitochondrial dynamics are three conserved dynamin-related GTPases (Kageyama et al., 2011). In mammals, mitochondrial fusion is mediated by mitofusion 1 and 2, and Opal, which are located in the outer and inner membranes, respectively. Mitochondrial division is mediated by Drpl, 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 AUTOPHAGIES

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

Allophagy

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

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

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

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

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

The embryo of the Caenorhabditis elegans nematode has been extensively used as an experimental model for exploring the role of autophagy in the degradation of paternal organelles (Al Rawi et al., 2012). It has been shown that paternal mitochondrial degradation depends on the formation of autophagosomes a few minutes after fertilization. This macroautophagic process is preceded by an active ubiquitination of some spermatozoon-inherited organelles, including mitochondria. The signal for such degradation is polyubiquitination of paternal mitochondria. Sato and Sato (2012) have also reported selective allophagy in such embryos.
It should be noted that the elimination of paternal mitochondrial DNA is not universal. Paternal inheritance of mitochondrial DNA, for example, has been reported in sheep and lower primates (St. John and Schatten, 2004; Zhao et al., 2004). A recent study using mice carrying human mitochondrial DNA indicated that this DNA was transmitted by males to the progeny in four successive generations, confirming the paternal transmission of mitochondrial DNA (Kidgotko et al., 2013). Apparently, human mitochondrial DNA safely passed via the male reproductive tract of several mice in several generations. This and a few other studies invoke a question regarding the existence of a specific mechanism responsible for paternal mitochondrial DNA transmission. Another pertinent, more important, unanswered question is: why are paternal mitochondria and/or their DNA eliminated from embryos? One hypothesis is that paternal mitochondria are heavily damaged by ROS prior to fertilization, and need to be removed to prevent potentially deleterious effects in the next generation (Sato and Sato, 2013).

Glycophagy

The delivery of glycogen to lysosomes for degradation is termed glycophagy. Three types of enzymes convert glucose into 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, diacylglycerol, 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 C++ load. It is concluded that EGCG induces lipophagy through a reduction in the accumulation of lipid droplets in endothelial cells. It is known that impairment of the lysosomal degradation process reduces autophagic flux leading to serious disorders in cardiovascular and metabolic tissues (Singh and Cuervo, 2011).

The following questions still remain to be answered and 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 damaged mitochondria, mitophagy) as well as LAMP-2 (a marker for autolysosomes) were expressed in these rats. This information provides clear evidence of ethanol toxicity because of the accumulation of lipid droplets in the cytoplasm of hepatocytes involving lipogenesis and lipolysis. Elevated levels of lipophagy and mitophagy reduce hepatocyte cell death under acute ethanol toxicity (Ding et al., 2011).
In conclusion, the enhanced autophagic sequestration of accumulated lipid droplets 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.

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, and 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.
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

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

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

Morphologically, during nucleophagy, a nuclear bleb containing the nuclear cargo is pinched off from the nucleus and directly engulfed and sequestered into an invagination of the vacuolar membrane rather than packaged into autophagosome-like vesicles. It has been shown that upon nitrogen starvation the initiation of piecemeal micronucleophagy of the nucleus (PMN) occurs, as stated above, at the nucleus–vacuole junction between the outer nuclear membrane protein, Nvj1p, and the vacuolar membrane protein, Vac8p. Recently, it was demonstrated that induction of PMN can be detected as early as after 3h of nitrogen starvation (Mijaljica *et al.*, 2012). Mijaljica and co-workers 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 – were required for PMN. In spite of the known molecular mechanisms discussed above, the specific conditions under which various cell nucleus components such as nucleoli, chromosomes, chromatin, histones, nuclear pore complexes, and nucleoplasm are degraded are not known.

**Pexophagy**

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

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

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

Reticulophagy

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

The ER stress signal, along with other signals (e.g., oxidative signal), is involved in autophagy. The former is involved in membrane formation and fusion, including autophagosome formation, autophagosome-lysosome 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 the ER stress. ER stress occurs under various physiological and pathological conditions where the capacity of the ER to fold proteins becomes saturated, for example as a response to incompetent or aggregation prone proteins, Ca\textsuperscript{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).

**Ribophagy**

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

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

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

It is important to identify the genes required for ribophagy. Kraft et al. (2008) indicated the involvement of two proteins, ubiquitin-specific protease 3 (Ubp3) enzyme and Ubp3-associated cofactor (Bres) in the selective degradation of ribosomes, but not for bulk autophagy. They also indicated that ribophagy affects the entire 60S subunit, but not the 40S subunit, suggesting differential degradation of large and small subunits. These authors, furthermore, demonstrated the involvement of Atg1 and Atg7 in the transport of ribosomes to the vacuole in the yeast *S. cerevisiae*. It also has been reported that the Ubp3/Bre5 complex interacts with Atg19 protein and modulates its ubiquitination.
It is concluded that ribosome degradation relies on both ribophagy and non-selective autophagy. The evidence presented there and from other studies confirms a cross-talk between selective autophagy and ubiquitin-dependent processes. The majority of cellular proteins and most other cell components are eventually degraded and recycled in a cell either by autophagy or the ubiquitin–proteasome pathway or by a combination of these two systems. In fact, there is a connection between autophagy and ubiquitin modification and destruction by the proteasome pathways of protein degradation.

Xenophagy

The successful invasion of the host cell by pathogenic microorganisms depends on their ability to subvert intracellular signaling to avoid triggering the cell’s immune response. The host cell, under normal conditions, possesses pathways (xenophagy) that protect it from infection. Post-translation modifications (ubiquitination) play a role in the activation of xenophagy. A link between ubiquitination and the regulation of autophagy has been established (Dupont et al., 2010). It is also known that p62 proteins target protein aggregates for degradation via autophagy. Pathogens, however, have developed mechanisms that subvert the cell’s defense systems (xenophagy), replicating themselves. Mycobacterium tuberculosis, for example, prevents inflammasome activation (Master et al., 2008). Other mechanisms involve 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 the digestive enzymes in the acinar cells, the result could be pancreatitis. Under normal physiological conditions selective autophagy (zymophagy) degrades the activated zymogen granules, avoiding the release of digestive enzymes into the cytoplasm and thus preventing further trypsinogen activation and cell death. In other words, zymophagy has a critical function in secretory homeostasis and cell response to injury by selective degradation of altered secretory granules in acute pancreatitis.

In conclusion, zymophagy protects the pancreas from self-digestion. It is a selective form of autophagy, a cellular process to specifically detect and degrade secretory granules
containing activated enzymes before they can digest the organ (Vaccaro, 2012). Zymophagy is activated in pancreatic acinar cells during pancreatitis-induced vesicular transport alteration to sequester and degrade potentially deleterious, activated zymogen granules.

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