Autophagy: the powerful of the immune response

Book · December 2017

CITATIONS
0

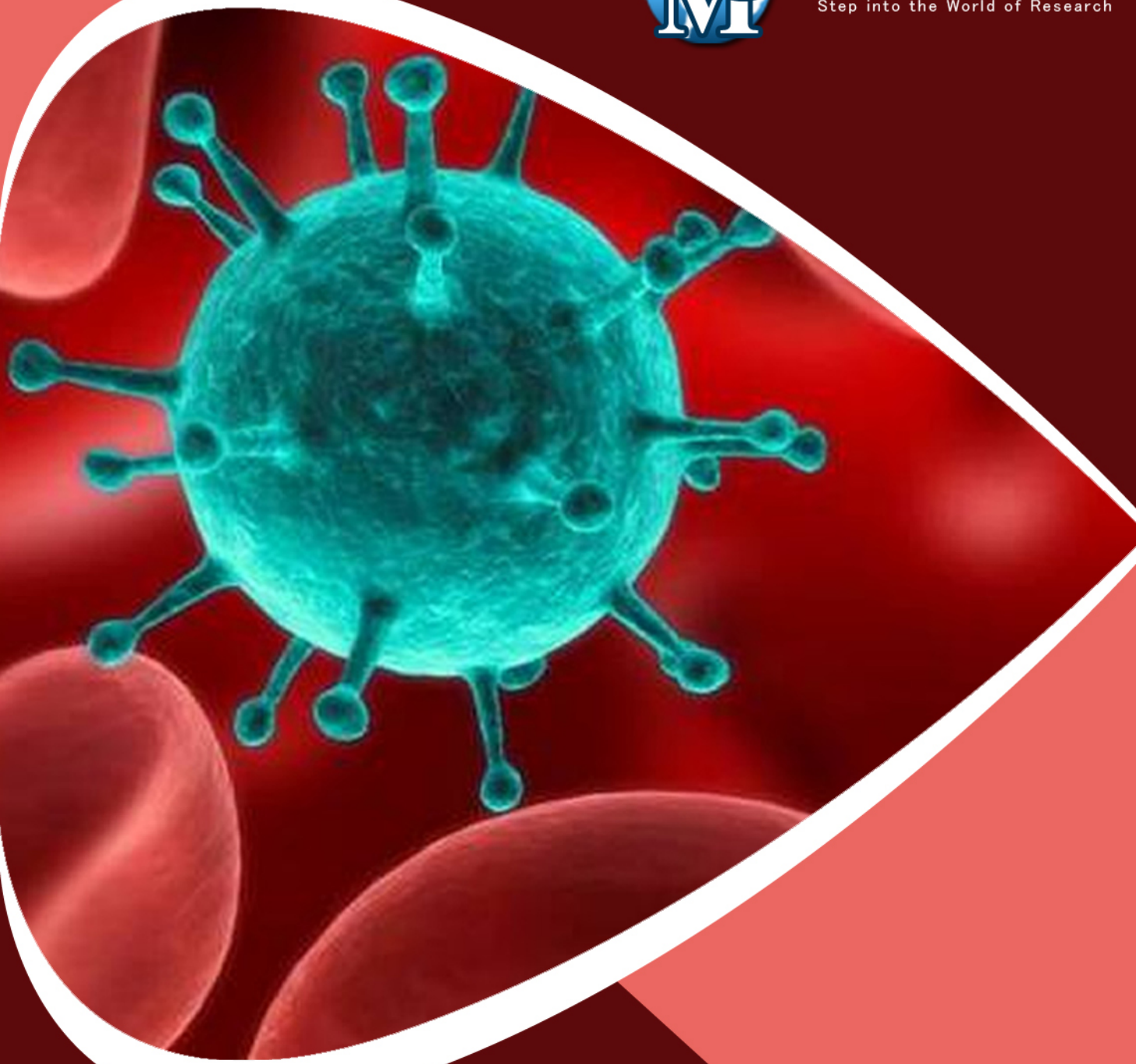
READS
156

1 author:

Mosab Nouraldein Mohammed Hamad
Elsheikh Abdallah Elbadri University
348 PUBLICATIONS **19** CITATIONS

SEE PROFILE

MedCrave
Step into the World of Research



Autophagy: The Powerful of Immune Response

Mosab Nouraldein

Autophagy: The Powerful of Immune Response

ABOUT THE AUTHORS

Mosab Nouraldein Mohammed Hamad*

Department of Parasitology and Medical Entomology, University of Elsheikh Abdallah Elbadri, Sudan

*Corresponding author:

Mosab NM Hamad, Department of Parasitology and Medical Entomology, University of Elsheikh Abdallah Elbadri, Sudan,
Email: musab.noor13@gmail.com

Published By:

MedCrave Group LLC

December 29, 2017

Contents

Dedication

To the soul of my brother Abdel Rahman Nouraldein Mohammed Hamad

Acknowledgement

To my friends at faculty of medical laboratory sciences, Elrazi university, Sudan

Introduction

Autophagy is an intracellular degradation system that delivers cytoplasmic constituents to the lysosome. Despite its simplicity, recent progress has demonstrated that autophagy plays a wide variety of physiological and pathophysiological roles, which are sometimes complex. Autophagy consists of several sequential steps sequestration, transport to lysosomes, degradation, and utilization of degradation products and each step may exert different function. This process is quite distinct from endocytosis-mediated lysosomal degradation of extracellular and plasma membrane proteins. There are three types of autophagy macroautophagy, micro autophagy, and chaperone-mediated autophagy and the term "autophagy" usually indicates macroautophagy unless otherwise specified. Autophagy is mediated by a unique organelle called the autophagosome. As autophagosomes engulf a portion of cytoplasm, autophagy is generally thought to be a nonselective degradation system. This feature is in marked contrast to the ubiquitin-proteasome system, which specifically recognizes only ubiquitinated proteins for proteasomal degradation. It is therefore reasonable to assume that the ubiquitin-proteasome system has numerous specific functions because it can selectively degrade thousands of substrates. Recent studies have clearly demonstrated that autophagy has a greater variety of physiological and pathophysiological roles than expected, such as starvation adaptation, intracellular protein and organelle clearance, development, anti-aging, elimination of microorganisms, cell death, tumor suppression, and antigen presentation. Additionally, in some situations, the contribution of autophagy seems to be very complicated. For example, it is very difficult to generalize the role of autophagy in cancer and cell death [1].

Macroautophagy (hereafter autophagy), or 'self-eating', is a conserved cellular pathway that controls protein and organelle degradation, and has essential roles in survival, development and homeostasis. Autophagy is also integral to human health and is involved in physiology, development, lifespan and a wide range of diseases, including cancer, neurode generation and microbial infection. Although research on this topic began in the late 1950s, substantial progress in the molecular study of autophagy has taken place during only the past 15 years. This review traces the key findings that led to our current molecular understanding of this complex process. The term 'autophagy' comes from the Greek words 'phagy' meaning eat, and 'auto' meaning self. Autophagy is an evolutionarily conserved process in eukaryotes by which cytoplasm cargo sequestered inside double-membrane vesicles are delivered to the lysosome for degradation. When autophagy was initially discovered more than 40 years ago, it was perplexing as to why the cell would self-digest its own components. The simplest hypothesis was that autophagy serves as a cellular rubbish-disposal mechanism. However, we have since learnt that this 'self-eating' process not only rids the cell of intracellular misfolded or long-lived proteins, superfluous or damaged organelles, and invading microorganisms, but also is an adaptive response to provide nutrients and energy on exposure to various stresses. Autophagy has been connected to human pathophysiology, and continued expansion of our knowledge about autophagy has had implications for fields as wide-ranging as cancer, neurodegeneration, immune response, development and ageing. This timeline reviews the history of autophagy research with a focus on the key events that occurred over the past 15 years, when our molecular understanding of this process first began.

History of autophagy

More than four decades ago, Clark and Novikoff observed mitochondria from mouse kidneys within membrane-bound compartments termed 'dense bodies', which were subsequently shown to include lysosomal enzymes. Ashford and Porter later observed membrane-bound vesicles containing semi-digested mitochondria and endoplasmic reticulum in the hepatocytes of rats that had been exposed to glucagon, and Novikoff and Essner observed that the same bodies contained lysosomal hydrolases. One year later, in 1963, at the Ciba Foundation symposium on lysosomes, de Duve founded the field when he coined the term 'autophagy' to describe the presence of single- or double-membrane vesicles that contain parts of the cytoplasm and organelles in various states of disintegration. He pointed out that these sequestering vesicles, or 'autophagosomes', were related to lysosomes and occurred in normal cells. The origin of the membrane surrounding the autophagosome is still controversial; de Duve suggested that the sequestering membranes are derived from preformed membranes, such as smooth endoplasmic reticulum. Cellular autophagy is observed in normal rat liver cells, but is enhanced in the livers of starved animals6, and in 1967 de Duve and Deter confirmed that glucagon induces autophagy. Ten years later, Pfeifer demonstrated the converse that insulin inhibits autophagy. Pioneering work by Mortimore and Schworer further demonstrated that amino acids, which are the end products of autophagic degradation, have an inhibitory effect on autophagy in rat liver cells. These early lines of evidence are consistent with our current understanding of autophagy as an adaptive catabolic and energy-generating process. Subsequently, Seglen and Gordon carried out the first biochemical analysis of autophagy and identified the pharmacological reagent 3-methyladenine as an autophagy inhibitor; they also provided the first evidence that protein kinases and phosphatases can regulate autophagy.

These early studies of autophagy from the 1950s to the early 1980s were based on morphological analyses. de Duve and others primarily examined the terminal stages of the process, the steps just before or after fusion with the lysosome. Subsequent studies by Seglen's laboratory began to use electro-injected radioactive probes to examine the early and intermediate steps of autophagy, leading to the identification of the phagophore (the initial sequestering vesicle that develops into the autophagosome), as well as the amphisome (a non-lysosomal vesicle formed by

the fusion of autophagosomes and endosomes). As early as the 1960s, de Duve suggested that most, if not all, living cells must employ a mechanism for nonspecific bulk segregation and digestion of portions of their own cytoplasm in the lysosome5, but also hinted at the need of a selective proteolytic mechanism acting on abnormal cellular proteins or organelles. In 1973, Bolender and weibel provided some of the first evidence that a specific organelle (the smooth endoplasmic reticulum) can be engulfed by autophagy. Four years later, Beaulaton and Lockshin suggested that mitochondria are selectively cleared during insect metamorphosis. In 1983, Veenhuis demonstrated that superfluous peroxisomes are selectively degraded by autophagy in the yeast Hansenula polymorpha15, and five years later Lemasters and colleagues showed that changes in mitochondrial membrane potential lead to the onset of autophagy16. Further evidence that autophagy can be selective was provided by subsequent studies in yeast and higher eukaryotes [2].

Mechanism of Autophagy

The term 'autophagy', derived from the Greek meaning 'eating of self', was first coined by Christian de Duve over 40 years ago, and was largely based on the observed degradation of mitochondria and other intra-cellular structures within lysosomes of rat liver perfused with the pancreatic hormone, glucagon. The mechanism of glucagon-induced autophagy in the liver is still not fully understood at the molecular level, other than that it requires cyclic AMP induced activation of protein kinase-A and is highly tissue-specific. In recent years the scientific world has 'rediscovered' autophagy, with major contributions to our molecular understanding and appreciation of the physiological significance of this process coming from numerous laboratories. Although the importance of autophagy is well recognized in mammalian systems, many of the mechanistic breakthroughs in delineating how autophagy is regulated and executed at the molecular level have been made in yeast (Saccharomyces cerevisiae). Currently, 32 different autophagy-related genes (Atg) have been identified by genetic screening in yeast and, significantly, many of these genes are conserved in slime mould, plants, worms, flies and mammals, emphasizing the importance of the autophagic process in responses to starvation across phylogeny. There are three defined types of autophagy: macro-autophagy, micro-autophagy, and chaperone-mediated autophagy, all of which promote proteolytic degradation of cytosolic components at the lysosome. Macro-autophagy delivers cytoplasmic cargo to the lysosome through the intermediary of a double membrane-bound vesicle, referred to as an autophagosome that fuses with the lysosome to form an autolysosome. In micro-autophagy, by contrast, cytosolic components are directly taken up by the lysosome itself through invagination of the lysosomal membrane. Both macro- and micro-autophagy are able to engulf large structures through both selective and non-selective mechanisms. In chaperone-mediated autophagy (CMA), targeted proteins are translocated across the lysosomal membrane in a complex with chaperone proteins (such as Hsc-70) that are recognized by the lysosomal membrane receptor lysosomal-associated membrane protein 2A (LAMP-2A), resulting in their unfolding and degradation. Due to recent and increased interest specifically in macroautophagy and its role in disease, this review focuses on molecular and cellular aspects of macro-autophagy (henceforth referred to as 'autophagy') and how it is regulated under both healthy and pathological conditions.

Basic autophagy mechanism

Autophagy begins with an isolation membrane, also known as a phagophore that is likely derived from lipid bilayer contributed by the endoplasmic reticulum (ER) and/or the trans-Golgi and endosomes, although the exact origin of the phagophore in mammalian cells is controversial. This phagophore expands to engulf intra-cellular cargo, such as protein aggregates, organelles and ribosomes, thereby sequestering the cargo in a double-membraned autophagosome. The loaded autophagosome matures through fusion with the lysosome, promoting the degradation of autophagosomal contents by lysosomal acid proteases. Lysosomal permeases and transporters export amino acids and other by-products of degradation back out to the cytoplasm, where they can be re-used for building macromolecules and for metabolism. Thus, autophagy may be thought of as a cellular 'recycling factory' that also promotes energy efficiency through ATP generation and mediates damage control by removing non-functional proteins and organelles. There are five key stages :

I. phagophore formation or nucleation;

II. Atg5-Atg12 conjugation, interaction with Atg16L and multi-merization at the phagophore;

III. LC3 processing and insertion into the extending phagophore membrane;

IV. capture of random or selective targets for degradation; and

V. fusion of the autophagosome with the lysosome, followed by proteolytic degradation by lysosomal proteases of engulfed molecules.

Phagophore formation is under the control of multiple signalling events

Phagophore membrane formation in yeast is formed at, or organized around, a cytosolic structure known as the pre-autophagosomal structure (PAS), but there is no evidence for a PAS in mammals. In mammalian cells, phagophore membranes appear to initiate primarily from the ER in dynamic equilibrium with other cytosolic membrane structures, such as the trans-Golgi and late endosomes and possibly even derive membrane from the nuclear envelope under restricted conditions. However, given the relative lack of transmembrane proteins in autophagosomal membranes, it is not yet possible to completely rule out de novo

membrane formation from cytosolic lipids in mammalian cells. The activity of the Atg1 kinase in a complex with Atg13 and Atg17 is required for phagophore formation in yeast, possibly by regulating the recruitment of the transmembrane protein Atg9 that may act by promoting lipid recruitment to the expanding phagophore. This step is regulated by the energy-sensing TOR kinase that phosphorylates Atg13, preventing it from interacting with Atg1 and rendering initiation of autophagy sensitive to growth factor and nutrient availability. Ulk-1, a mammalian homologue of Atg1 is critical for autophagy in maturing reticulocytes but it remains to be determined whether Ulk-1, or indeed Ulk-2 (a second Atg1 homologue), functions analogously in promoting autophagy in mammalian systems. These early steps in phagophore formation in mammalian systems are an area that requires greater investigation and is likely to lead to many important findings, given that these processes are tightly regulated in yeast and are a nexus for signalling input in higher systems. The role of class III PI-3 kinases, notably Vps34 (vesicular protein sorting 34) and its binding partner Atg6/Beclin-1, in phagophore formation and autophagy is relatively well understood in mammalian systems. Vps34 is involved in various membrane-sorting processes in the cell but is selectively involved in autophagy when complexed to Beclin-1 and other regulatory proteins. Vps34 is unique amongst PI3-kinases in only using phosphatidylinositol (PI) as substrate to generate phosphatidyl inositol triphosphate (PI3P), which is essential for phagophore elongation and recruitment of other Atg proteins to the phagophore. The interaction of Beclin-1 with Vps34 promotes its catalytic activity and increases levels of PI3P, but how this is regulated in response to starvation signalling is not yet resolved.

Beclin-1 is mono-allelically deleted in human breast, ovarian and prostate cancer, leading various cancer biologists to suggest that autophagy has tumor-suppressor properties. Consistently, while Beclin-1 null mice are embryonic lethal, Beclin-1 heterozygous mice are predisposed to lymphoma, hepato-cellular carcinoma and other cancers. Autophagy has been postulated to prevent tumorigenesis by limiting necrosis and inflammation, inducing cell cycle arrest and preventing genome instability. Autophagy has also recently been shown to be required for key aspects of the senescent cell phenotype, which is known to be anti-tumorigenic. However, as a cell survival mechanism, others have argued that autophagy may promote drug resistance and tumour cell adaptation to stress. Ultimately, the role of autophagy in cancer may be cell type- and/or stage-specific. Additional regulatory proteins complex with Vps34 and Beclin-1 at the ER and nucleated phagophore to either promote autophagy, such as UVRAG, BIF-1, Atg14L and Ambra, or to inhibit autophagy, such as Rubicon and Bcl-2. Like Beclin-1, UVRAG has been shown to be mono-allelically deleted in human cancer. The precise subunit composition of complexes at the ER containing Vps34 and Beclin-1 is determined by signaling events in the cell that remain to be fully elucidated but, in many instances, are sensitive to nutrient availability in the microenvironment. One well-characterized regulatory event is the interaction of Beclin-1 with Bcl-2, which disrupts the interaction of Beclin-1 with Vps34. Thus, Beclin-1 activity in autophagy is inhibited by interaction with Bcl-2 (and Bcl-XL) at the ER. This interaction is mediated by the BH3 domain in Beclin-1 and disrupted by Jnk1-mediated phosphorylation of Bcl-2 in response to starvation-induced signalling, thereby allowing autophagy to proceed. Thus, Bcl-2 plays a dual role in determining cell viability that may depend on its subcellular localization: (a) a pro-survival function at mitochondria inhibiting cytochrome c release, thereby blocking apoptosis; and (b) an autophagy-inhibitory activity at the ER, mediated by interaction with Beclin1 that can lead to non-apoptotic cell death. The crosstalk between autophagy and apoptosis extends beyond the regulation of Beclin1 and Bcl-2. For example, calpain-mediated cleavage of Atg5 blocked its activity in autophagy, caused it to translocate to the mitochondria, where its interaction with Bcl-XL resulted in cytochrome c release, caspase activation and apoptosis. How the balance between autophagy and apoptosis is determined in the cellular response to specific stresses is a research area of extreme interest given its relevance for disease progression and treatment, but again is an area that is not resolved.

Atg5-Atg12 conjugation

There are two ubiquitin-like systems that are key to autophagy acting at the Atg5-Atg12 conjugation step and at the LC3 processing step (see below). In the first of these systems, Atg7 acting like an E1 ubiquitin activating enzyme activates Atg12 in an ATP-dependent manner by binding to its carboxyterminal glycine residue. Atg12 is then transferred to Atg10, an E2-like ubiquitin carrier protein that potentiates covalent linkage of Atg12 to lysine 130 of Atg5. Conjugated Atg5-Atg12 complexes in pairs with Atg16L dimers to form a multimeric Atg5- Atg12-Atg16L complex that associates with the extending phagophore. The association of Atg5-Atg12-Atg16L complexes is thought to induce curvature into the growing phagophore through asymmetric recruitment of processed LC3B-II. Atg5-Atg12 conjugation is not dependent on activation of autophagy and once the autophagosome is formed, Atg5-Atg12-Atg16L dissociates from the membrane, making conjugated Atg5-Atg12 a relatively poor marker of autophagy. Interestingly, genome-wide association studies (GWAS) linked a mutation (T330A) in ATG16L to Crohn's disease, a progressive inflammatory bowel disease in humans. Loss of functional Atg16L in mice blocked autophagy in intestinal Paneth cells, resulted in increased inflammasome activation and aberrant inflammatory cytokine production following challenge of Atg16L deficient macrophages with bacterial endotoxin, and reduced secretion of antimicrobial peptides from intestinal Paneth cells in Atg16L hypomorphic mice. Similar changes in Paneth cell granule production were observed in Crohn's patients with the ATG16L mutation and this is predicted to alter the diversity of gut microbiota. The IRGM locus was also linked to Crohn's disease by GWAS and, while the specific function of the IRGM GTPase in autophagic turnover of intra-cellular bacteria is not clear, reduced expression of IRGM in Crohn's disease appears

to be associated with the identified SNP in its upstream regulatory sequences.

LC3 processing

The second ubiquitin-like system involved in autophagosome formation is the processing of microtubule-associated protein light chain 3 (LC3B), which is encoded by the mammalian homologue of Atg8. LC3B is expressed in most cell types as a full-length cytosolic protein that, upon induction of autophagy, is proteolytically cleaved by Atg4, a cysteine protease, to generate LC3B-I. The carboxyterminal glycine exposed by Atg4-dependent cleavage is then activated in an ATP-dependent manner by the E1-like Atg7 in a manner similar to that carried out by Atg7 on Atg12. Activated LC3B-I is then transferred to Atg3, a different E2-like carrier protein before phosphatidylethanolamine (PE) is conjugated to the carboxyl glycine to generate processed LC3B-II. Recruitment and integration of LC3B-II into the growing phagophore is dependent on Atg5-Atg12 and LC3B-II is found on both the internal and external surfaces of the autophagosome, where it plays a role in both hemifusion of membranes and in selecting cargo for degradation. The synthesis and processing of LC3 is increased during autophagy, making it a key readout of levels of autophagy in cells. The related molecule, GABARAP [aminobutyric type A (GABAA) receptor associated protein] undergoes similar processing during autophagy and GABARAP-II co-localizes with LC3-II at autophagosomes. The significance of LC3-related molecules in autophagy is not clear, although it has been postulated that differences in their protein-protein interactions may determine which cargo is selected for uptake by the autophagosome.

Selection, or not, of cargo for degradation?

In general, autophagy has been viewed as a random process because it appears to engulf cytosol indiscriminately. Electron micrographs frequently show autophagosomes with varied contents, including mitochondria, ER and Golgi membranes. However, there is accumulating evidence that the growing phagophore membrane can interact selectively with protein aggregates and organelles. It is proposed that LC3B-II, acting as a 'receptor' at the phagophore, interacts with 'adaptor' molecules on the target (e.g. protein aggregates, mitochondria) to promote their selective uptake and degradation. The best-characterized molecule in this regard is p62/SQSTM1, a multi-functional adaptor molecule that promotes turnover of poly-ubiquitinated protein aggregates. Mutation of p62/SQSTM1 is linked to Paget's disease, in which abnormal turnover of bone results in bone deformation, arthritis and nerve injury. Osteoclasts in such individuals show deregulated NF-kB signalling and accumulation of ubiquitinated proteins consistent with a key role for autophagy in normal bone development and function. Other molecules, such as NBR1, function similarly to p62/SQSTM1 in promoting turnover of ubiquitinated proteins, while in yeast, Uth1p and Atg32 have been identified as proteins that promote selective uptake of mitochondria, a process known as mitophagy.

Fusion with the lysosome

When the autophagosome completes fusion of the expanding ends of the phagophore membrane, the next step towards maturation in this self-degradative process is fusion of the autophagosome with the specialized endosomal compartment that is the lysosome to form the 'autolysosome'. It has been variously suggested that fusion of the autophagosome with early and late endosomes, prior to fusion with the lysosome, both delivers cargo and also delivers components of the membrane fusion machinery and lowers the pH of the autophagic vesicle before delivery of lysosomal acid proteases. This aspect of the process is relatively understudied but requires the small G protein Rab7 in its GTP-bound state, and also the Presenilin protein that is implicated in Alzheimer's disease. The cytoskeleton also plays a role in autolysosome formation, since agents such as nocadazole, which are microtubule poisons, block fusion of the autophagosome with the lysosome. Within the lysosome, cathepsin proteases B and D are required for turnover of autophagosomes and, by inference, for the maturation of the autolysosome. Lamp-1 and Lamp-2 at the lysosome are also critical for functional autophagy, as evidenced by the inhibitory effect of targeted deletion of these proteins in mice on autolysosome maturation. Interestingly, inactivation of LAMP-2 is the causative genetic lesion associated with Danon disease in humans, an X-linked condition that causes cardiomyocyte hyper-trophy and accumulation of autophagosomes in heart muscle. Similar cardiac defects are observed in Lamp-2-null mice, as well as skeletal abnormalities and periodontitis associated with inflammation arising from a failure to eliminate intracellular pathogens in the oral mucosa.

Atg5/Atg7-independent autophagy

Although Atg5- and Atg7-dependent autophagy has been shown to be critical for survival during the starvation period in the first few days immediately following birth, recent evidence has identified an alternative Atg5/Atg7-independent pathway of autophagy. This pathway of autophagy was not associated with LC3 processing but appeared to specifically involve autophagosome formation from late endosomes and the trans-Golgi. Atg7-independent autophagy had been implicated in mitochondrial clearance from reticulocytes, and it has consistently been shown that Ulk-1 (a mammalian homologue of Atg1) is required for both reticulocyte clearance of mitochondria and, along with Beclin-1, for Atg5/Atg7-independent autophagy. The exact molecular basis of Atg5/Atg7-independent autophagy remains to be elucidated.

Selective autophagy

Here, we focus in more depth on selective autophagy, given its significance for neuropathies, cancer and heart disease. As briefly mentioned above, p62/SQSTM1 associates with polyubiquitinated proteins and aggregates through its ubiquitin-binding domain (UBD), with LC3B-II through its LC3-interacting Region (LIR), but also regulates NF-κB

signalling through interaction with Traf-6. When autophagy is defective, as in mice with targeted deletion of Atg7 , p62-associated poly-ubiquitinated aggregates accumulated in cells and the combined knockout of Atg7 and p62 was observed to 'rescue' the accumulation of these aberrant cytosolic inclusions. p62 is the major constituent of Mallory bodies in the liver that accumulate in human hepatocellular carcinoma, where recent work indicates that elevated p62 levels play an active role in deregulating NF-κB signalling and inducing inflammation-associated tumorigenesis. Intracellular aggregate accumulation plays a particularly significant role in the etiology of neurodegenerative diseases, including dementia, Alzheimer's, Huntington's, Parkinson's and Creutzfeldt-Jakob/prion diseases. For example, polyglutamine-expansion repeats, as seen in mutant huntingtin (Huntington's disease), mutant forms of α-synuclein (familial Parkinson's disease) and different forms of tau (Alzheimer's disease) are dependent on autophagy for their clearance from neurons. Consistently, neuronal-specific inactivation of the key autophagy genes Atg5 or Atg7 results in intracellular aggregate accumulation and neurodegeneration in mice. This relatively recent link between autophagy and neuropathies has prompted interested in the development of autophagy-inducing drugs to treat these debilitating diseases. Autophagy-dependent degradation of mitochondria, termed mitophagy, is important for maintaining the integrity of these critical organelles and limiting the production of reactive oxygen species. The first protein identified to be involved in mitophagy was Uth1p, a yeast protein that is required for mitochondrial clearance by autophagy, but it is unknown how Uth1 interacts with the autophagosome and mediates mitophagy, and there are no known mammalian homologues. More recently, Atg32, a mitochondria-anchored protein, was found to be required for mitophagy in yeast, where it functions through interaction with Atg8 and Atg11, suggesting that it functions as a mitochondrial receptor for mitophagy. Atg32, like Uth1, has no known homologues in mammals, but contains an amino acid motif, WXXI, that is required for interaction with Atg8 and Atg11 and is conserved in the LIR of p62. Other molecules that are implicated in mitophagy are BNIP3L, which is involved in mitochondrial clearance in differentiating red blood cells, Ulk-1, which is the mammalian homologue of Atg1, and Parkin, encoded by a gene that is genetically linked to Parkinson's disease. Parkin is an E3 ubiquitin ligase that is located at the outer mitochondrial membrane, suggesting that key molecules at the mitochondria require to be ubiquitinated in order to promote the uptake of mitochondria by autophagosomes.

Both peroxisomes and ribosomes are selectively eliminated via autophagy in yeast. Methylotrophic yeasts use micropexophagy (direct engulfment by the vacuole) and macropexophagy (autophagosome-mediated delivery to the vacuole) to remove peroxisomes during adaptation to an alternative energy source in which Atg30 was essential as an adaptor interacting with peroxisome proteins (Pex3 and Pex14) and with the autophagosome (Atg11 and Atg17) . Ribosomes are also selectively degraded during starvation (ribophagy), a process that is dependent on the catalytic activity of the Ubp3p/Bre5p ubiquitin protease. By comparison with yeast, these specialized forms of autophagy are under-studied in mammalian systems.

Signaling pathways that regulate autophagy

Autophagy is active at basal levels in most cell types where it is postulated to play a housekeeping role in maintaining the integrity of intracellular organelles and proteins. However, autophagy is strongly induced by starvation and is a key component of the adaptive response of cells and organisms to nutrient deprivation that promotes survival until nutrients become available again. How is autophagy induced in response to starvation signals? A major player in nutrient sensing and in regulating cell growth and autophagy is the target of rapamycin (TOR) kinase, which is a signalling control point downstream of growth factor receptor signalling, hypoxia, ATP levels and insulin signalling. TOR kinase is activated downstream of Akt kinase, PI3-kinase and growth factor receptor, signalling when nutrients are available and acting to promote growth through induction of ribosomal protein expression and increased protein translation. Importantly, TOR acts to inhibit autophagy under such growth-promoting conditions and, while this is mediated through its inhibitory effects on Atg1 kinase activity in yeast and Drosophila, it is not yet clear how this is carried out in mammalian cells. TOR kinase is repressed by signals that sense nutrient deprivation, including hypoxia. Upstream of TOR, activation of adenosine **5′**-monophosphate (AMP)-activated protein kinase (AMPK) in response to low ATP levels promotes the inhibitory activity of the Tsc1/Tsc2 tumour suppressor proteins on Rheb, a small GTase required for mTOR activity. Reduced Akt activity in response to reduced growth factor receptor activity also represses TOR kinase through Tsc1 and Tsc2, while TOR can be artificially inhibited by treatment of cells with rapamycin. Thus, reduced TOR activity induces autophagy, again ensuring that the cell adapts to its changing environment through reduced growth and increased catabolism. Based on these observations and that TOR lies downstream of oncogenes such as Akt, use of rapamycin has been tested in clinical trials for cancer therapy, where it is postulated to be act to inhibit tumour growth by blocking protein translation and by inducing autophagy. However, TOR can function as the catalytic component of two distinct complexes, known as TORC1 and TORC2, and rapamycin appears to have greater inhibitory activity against TORC1, driving the search for so-called 'rapalogs' that target both TORC1 and TORC2. As mentioned, hypoxia also activates autophagy through effects that are both dependent on target genes induced by hypoxia-inducible factor (HIF) and also through HIF-independent effects that are likely mediated through TOR inhibition downstream of AMPK, REDD1 and Tsc1/Tsc2. Given that hypoxia induces ER stress through the unfolded protein response, and that mitochondria have reduced function in oxidative phosphorylation under hypoxia, the induction of autophagy may allow the cell to eliminate portions of compacted ER and to reduce mitochondrial mass at a time when oxygen is not available to accept free electrons from the respiratory chain. This adaptive response

to hypoxia would prevent wasteful ATP consumption at the ER and limit production of reactive oxygen species at the mitochondria. Increased autophagy would also allow the cell to generate ATP from catabolism at a time when ATP production by oxidative phosphorylation is limited.

Specific HIF targets in autophagy include BNIP3 and BNIP3L that are non-canonical members of the Bcl-2 superfamily of cell death regulators. Although linked to cell death, the normal function of these proteins appears to be in mitophagy. As discussed, BNIP3L/NIX plays a physiological role in mitochondrial clearance from maturing reticulocytes, while BNIP3 has a similar role in cardiac and skeletal muscle in response to oxidative stress. The extent to which BNIP3 and BNIP3L are functionally redundant is not resolved and differential regulation of their expression may explain aspects of their non-redundancy *in vivo*. Various models have been proposed to explain how BNIP3/BNIP3L function in mitophagy, including a role for BNIP3 in derepressing Beclin-1 through disruption of its interaction with Bcl-2. However, a more direct role for BNIP3L in promoting mitochondrial clearance through interaction with the LC3-related molecule GABARAP has also been demonstrated, while BNIP3 interacts with Rheb, suggesting an additional indirect role in hypoxia-induced autophagy. Autophagy is known to induce cell cycle arrest and, while it appears that this may be largely driven by nutrient deprivation-induced inhibition of TOR activity and downstream effects on translation of key cell cycle genes, such as cyclin D1, it is not clear whether autophagy can induce cell cycle arrest independent of TOR signalling. This is an area of research that will likely be of increased interest moving forward, given its importance to understanding how and at what stages autophagy acts in tumour progression [3].

Autophagy and Diabetes

Autophagy and mitochondria in obesity and type 2 diabetes

Obesity and type 2 diabetes are growing health problems worldwide. The three principal diabetogenic factors are adiposity, insulin resistance in skeletal muscle, and decreased insulin production by pancreatic cells. During recent years, macroautophagy (hereafter autophagy)-sequestration and lysosomal degradation of cellular components - has emerged as an important player in these processes, playing a protective role against development of insulin resistance and diabetes of particular importance is the removal of dysfunctional mitochondria via mitophagy, a form of macroautophagy selective for mitochondria. Both muscle insulin resistance and -cell dysfunction largely depend on metabolic overload of mitochondria, which results in incomplete -oxidation, oxidative stress, accumulation of toxic lipid intermediates, and mitochondrial damage. Mitophagy eliminates this vicious cycle of oxidative stress and mitochondrial damage, and thus counteracts pathogenic processes. Autophagy also mediates exercise-induced increases in muscle glucose uptake and protects cells against ER stress in diabetogenic conditions. On the other hand, adipose tissue autophagy promotes adipocyte differentiation, possibly through its role in mitochondrial clearance. Being involved in many aspects, autophagy appears to be an attractive target for therapeutic interventions against obesity and diabetes [4].

The role of autophagy in the pathophysiology of diabetes mellitus

An emerging body of evidence supports a role for autophagy in the pathophysiology of type 1 and type 2 diabetes mellitus. Persistent high concentrations of glucose lead to imbalances in the antioxidant capacity within the cell resulting in oxidative stress-mediated injury in both disorders. An anticipated consequence of impaired autophagy is the accumulation of dysfunctional organelles such as mitochondria within the cell. Mitochondria are the primary site of the production of reactive oxygen species (ROS), and an imbalance in ROS production relative to the cytoprotective action of autophagy may lead to the accumulation of ROS. Impaired mitochondrial function associated with increased ROS levels have been proposed as mechanisms contributing to insulin resistance. In this article we review and interpret the literature that implicates a role for autophagy in the pathophysiology of type 1 and type 2 diabetes mellitus as it applies to -cell dysfunction, and more broadly to organ systems involved in complications of diabetes including the cardiovascular, renal and nervous systems [5].

Role of autophagy in diabetes and mitochondria

Type 2 diabetes mellitus is characterized by insulin resistance and failure of pancreatic beta-cells producing insulin. Mitochondrial dysfunction may play a role in both processes of diabetes. Autophagy maintains cellular homeostasis through degradation and recycling of organelles such as mitochondria. As dysfunctional mitochondria are the main organelles removed by autophagy, we studied the role of autophagy in diabetes using mice with beta-cell-specific deletion of the Atg7 gene. Atg7-mutant mice showed reduction in beta-cell mass and pancreatic insulin content. Electron microscopy showed swollen mitochondria and other Ultrastructural changes in autophagy-deficient beta-cells. Insulin secretory function ex vivo was also impaired. As a result, Atg7-mutant mice showed hypoinsulinemia and hyperglycemia. These results suggest that autophagy is necessary to maintain structure, mass, and function of beta-cells. Besides its effect on beta-cells, autophagy may affect insulin sensitivity because mitochondrial dysfunction has been implicated in insulin resistance and autophagy is involved in the maintenance of the organelles. Furthermore, since aging is associated with impaired glucose tolerance, decline of autophagic activity may be involved in age-associated reduction of glucose tolerance [6].

Autophagy in diabetes: β-cell dysfunction, insulin resistance, and complications

Autophagy functions to degrade and recycle intracellular proteins and damaged organelles, maintaining the normal

cellular function. Autophagy has been shown to play an important role in regulating normal function of pancreatic cells and insulin-target tissues, such as skeletal muscle, liver, and adipose tissue. Enhanced autophagy also acts as a protective mechanism against oxidative stress in these tissues. Altered autophagic activity has been implicated in the progression of obesity to type 2 diabetes through impaired -cell function and development of insulin resistance. In this review, we outline the normal regulation of autophagy in cells and insulin target tissues and explore the dysregulation of autophagy in diabetic animal models and human subjects with type 2 diabetes. Furthermore, we highlight the role of impaired autophagy in the pathophysiology of diabetic complications, including nephropathy and cardiomyopathy. Finally, we summarize how autophagy might be targeted as a therapeutic option in type 2 diabetes [7].

The role of autophagy in the pathogenesis of diabetic nephropathy

Diabetic nephropathy is a leading cause of end-stage renal disease worldwide. The multipronged drug approach targeting blood pressure and serum levels of glucose, insulin, and lipids fails to fully prevent the onset and progression of diabetic nephropathy. Therefore, a new therapeutic target to combat diabetic nephropathy is required. Autophagy is a catabolic process that degrades damaged proteins and organelles in mammalian cells and plays a critical role in maintaining cellular homeostasis. The accumulation of proteins and organelles damaged by hyperglycemia and other diabetes-related metabolic changes is highly associated with the development of diabetic nephropathy. Recent studies have suggested that autophagy activity is altered in both podocytes and proximal tubular cells under diabetic conditions. Autophagy activity is regulated by both nutrient state and intracellular stresses. Under diabetic conditions, an altered nutritional state due to nutrient excess may interfere with the autophagic response stimulated by intracellular stresses, leading to exacerbation of organelle dysfunction and diabetic nephropathy. In this review, we discuss new findings showing the relationships between autophagy and diabetic nephropathy and suggest the therapeutic potential of autophagy in diabetic nephropathy [8].

Autophagy in pancreatic β-cells and its implication in diabetes: Autophagy is a conserved system for the degradation of cytoplasmic proteins and organelles. During insulin resistance, in which insulin secretion is enhanced and -cell mass is increased owing to changes in the expression and function of various proteins in pancreatic -cells, autophagic activity appears to also be enhanced to adapt to the dynamic changes occurring in - cells. Indeed, defective autophagy in -cells recapitulates several features that are observed in islets during the development of type 2 diabetes mellitus. In addition, the dysregulation of autophagic activity appears to occur in the -cells of type 2 diabetic model mice and type 2 diabetes mellitus patients. These lines of evidence suggest that autophagic failure may be implicated in the pathophysiology of type 2 diabetes mellitus. In this review, we summarized the recent findings regarding how autophagy in -cells is regulated and how dysfunction of the autophagic machinery may lead to the dysfunction of -cells [9].

Autophagy and mitophagy in diabetic cardiomyopathy: Diabetic cardiomyopathy is a heart muscle-specific disease that increases the risk of heart failure and mortality in diabetic patients independent of vascular pathology. Mitochondria are cellular power plants that generate energy for heart contraction and concurrently produce reactive oxygen species that, if unchecked, may damage the mitochondria and the heart. Elimination of damaged mitochondria by autophagy known as mitophagy is an essential process for maintaining normal cardiac function at baseline and in response to various stress and disease conditions. Mitochondrial structural injury and functional impairment have been shown to contribute to diabetic heart disease. Recent studies have demonstrated an inhibited autophagic flux in the hearts of diabetic animals. Surprisingly, the diminished autophagy appears to be an adaptive response that protects against cardiac injury in type 1 diabetes. This raises several questions regarding the relationship between general autophagy and selective mitophagy in the diabetic heart. However, autophagy may play a different role in the hearts of type 2 diabetic animals [10].

Autophagic adaptations in diabetic cardiomyopathy differ between type 1 and type 2 diabetes: Little is known about the association between autophagy and diabetic cardiomyopathy. Also unknown are possible distinguishing features of cardiac autophagy in type 1 and type 2 diabetes. In hearts from streptozotocin-induced type 1 diabetic mice, diastolic function was impaired, though autophagic activity was significantly increased, as evidenced by increases in microtubule-associated protein 1 light chain 3/LC3 and LC3-II/-I ratios, SQSTM1/p62 (sequestosome 1) and CTSD (cathepsin D), and by the abundance of autophagic vacuoles and lysosomes detected electron-microscopically. AMP-activated protein kinase (AMPK) was activated and ATP content was reduced in type 1 diabetic hearts. Treatment with chloroquine, an autophagy inhibitor, worsened cardiac performance in type 1 diabetes. In addition, hearts from db/db type 2 diabetic model mice exhibited poorer diastolic function than control hearts from db/+ mice. However, levels of LC3-II, SQSTM1 and phosphorylated MTOR (mechanistic target of rapamycin) were increased, but CTSD was decreased and very few lysosomes were detected ultrastructurally, despite the abundance of autophagic vacuoles. AMPK activity was suppressed and ATP content was reduced in type 2 diabetic hearts. These findings suggest the autophagic process is suppressed at the final digestion step in type 2 diabetic hearts. Resveratrol, an autophagy enhancer, mitigated diastolic dysfunction, while chloroquine had the opposite effects in type 2 diabetic hearts. Autophagy in the heart is enhanced in type 1 diabetes, but is suppressed in type 2 diabetes. This difference provides important insight into

the pathophysiology of diabetic cardiomyopathy, which is essential for the development of new treatment strategies [11].

The effects of autophagy on diabetic cardiomyopathy: Diabetes is a major predictor of heart failure. However, little is known regarding mechanisms how it causes cardiomyopathy. The purpose of this study was to determine whether prolonged exposure of cardiomyocytes to high glucose concentrations induces autophagy. For *in vitro* study, H9c2 cells were cultured with high glucose for 3 days. Cell viability was determined by trypan blue assay. Autophagic vacuoles were detected by MDC staining as well as immunoblot. For *in vivo* study, diabetes mellitus was induced by streptozotocin, 60mg/kg of body weight, intraperitoneally at 4-week-age in SD rats. Body weight and blood glucose level were monitored and sacrificed at 2 and 5 weeks. Deprived glucose as well as high glucose within medium induced a significant decrease of cell viability at 72hr. The conversion ratio of LC3 was significantly increased by high glucose at the same time. However, glucose deprivation dramatically converts LC3| to LC3|| at 12hr. Diabetic rats have shown the reduction of size of LV with growth retardation ($p<.05$) and higher level of LC3 protein expression, suggesting that autophagy was activated. Taken together, *in vitro* findings indicate that hyperglycemic oxidative stress is inducible to autophagy. *In vivo* studies show progression of pathological remodeling of heart development is associated with autophagy. Thereby, there might be potential role of autophagy in pathogenesis of diabetic cardiomyocytes [12].

Autophagy and mitophagy in diabetic cardiomyopathy

Diabetic cardiomyopathy is a heart muscle-specific disease that increases the risk of heart failure and mortality in diabetic patients independent of vascular pathology. Mitochondria are cellular power plants that generate energy for heart contraction and concurrently produce reactive oxygen species that, if unchecked, may damage the mitochondria and the heart. Elimination of damaged mitochondria by autophagy known as mitophagy is an essential process for maintaining normal cardiac function at baseline and in response to various stress and disease conditions. Mitochondrial structural injury and functional impairment have been shown to contribute to diabetic heart disease. Recent studies have demonstrated an inhibited autophagic flux in the hearts of diabetic animals. Surprisingly, the diminished autophagy appears to be an adaptive response that protects against cardiac injury in type 1 diabetes. This raises several questions regarding the relationship between general autophagy and selective mitophagy in the diabetic heart. However, autophagy may play a different role in the hearts of type 2 diabetic animals. In this review, we will summarize current knowledge in this field and discuss the potential functional roles of autophagy and mitophagy in the pathogenesis of diabetic cardiomyopathy. This article is part of a Special Issue entitled: Autophagy and protein quality control in cardiometabolic diseases [13].

Cardiac autophagy in diabetic cardiomyopathy

Autophagy is a process for bulk degradation and recycling of cytoplasmic components in lysosomes. A low level of constitutive autophagy is cytoprotective by maintaining the quality of proteins and organelles. It allows recycling of amino acids and removal of damaged organelles to eliminate oxidative stress and promote remodeling for survival. In the heart, autophagy plays an important role in cytoplasmic quality control and cardiac homeostasis under physiological and pathological conditions. Down-regulation of autophagy would cause abnormal proteins and organelles to accumulate, leading to apoptosis and cardiac dysfunction, the pathologies seen in diseases such as myocardial hypertrophy, cardiomyopathy, and ischemic heart disease. However, excessive induction of autophagy may destroy the cytosol and organelles and release apoptosis related factors, triggering cardiomyocyte death and impairing cardiac function. Thus, there is ongoing debate about whether up-regulated autophagy is the cause of cardiomyocyte death or whether it is actually an attempt to protect cells against cardiac stress conditions, including diabetes and ischemic heart disease.

Suppression of cardiac autophagy in type 1 diabetes

Recently, we have established the role of autophagy in the development of diabetic cardiomyopathy in type 1 diabetic animal models. At six months of age, OVE26 mice, an established type 1 diabetic mouse model generated through targeted over expression of calmodulin in cell, exhibit very high blood glucose concentrations, reduced serum insulin values, and elevated serum triglyceride levels, and they also exhibit cardiomyopathy characterized by clear morphological abnormalities and impaired cardiac performance. Evidence for diabetes-induced suppression of autophagic activity is uncovered by western blotting and electron microscopy, which demonstrate that diabetes decreases accumulation of lipidated microtubule-associated protein1 light chain 3 (LC3-II) and autophagosome formation in the heart. Moreover, streptozotocin (STZ) - induced diabetes also suppresses cardiac autophagy and impairs cardiac function. Mechanistically, AMP-activated protein kinase (AMPK) activity is significantly inhibited in diabetic OVE26 mice, chronic activation of AMPK by metformin restores cardiac autophagy in wild type diabetic hearts, but this effect is abolished in mice deficient of AMPKα2, indicating that AMPK regulates cardiac autophagy in diabetic cardiomyopathy. In addition, Diabetic hearts display activation of the tuberous sclerosis complex mammalian target of rapamycin (TSCmTOR) signaling pathway, as reflected by decreased phosphorylation of raptor, as well as increased phosphorylation of mTOR and its downstream effectors, 4 E binding protein 1 (4EBP1) and p70 ribosomal protein S6 kinase 1 (p70 S6K1). Activation of AMPK by metformin inhibits the TSC-mTOR pathway and restores cardiac autophagy in OVE26 mice. Finally, we demonstrate that AMPK activation attenuates diabetic cardiomyopathy through regulation of the switch between autophagy and

apoptotic machinery (He C, et al. [3] unpublished data). This effect is attributable to c-Jun N-terminal kinase (JNK)-mediated Bcl-2 (B-cell lymphoma 2) phosphorylation and subsequent Beclin1-Bcl-2 disassociation. In STZ-induced diabetic mice, hyperglycemia enhances the interaction between Beclin1 and Bcl-2 through inhibition of JNK1 and Bcl-2 phosphorylation and results in suppression of autophagy and induction of apoptosis. Activation of AMPK stimulates JNK1, which mediates Bcl-2 phosphorylation and subsequent Beclin1-Bcl-2 disassociation, leading to restoration of Cardiac autophagy and protection against cardiac apoptosis. As a result, cardiac structure and function are improved in diabetic mice. These data suggest that hyperglycemia suppresses cardiac autophagy, leading to cell death and cardiac dysfunction. Restoration of autophagy by activated AMPK prevents diabetic cardiomyopathy. However, a recent study demonstrates that high glucose directly inhibits autophagic flux in neonatal rat cardiomyocytes and in these cells the reduction of autophagy appears to be an adaptive response that functions to limit high glucose induced cardiomyocyte injury. Neonatal cardiomyocytes have been reported to behave substantially different from adult cardiomyocytes. Especially, autophagy is upregulated in the neonatal cardiac tissue during perinatal period of relative starvation. Thus, autophagy could be either protective or detrimental depending on the cell type and cellular environment.

Autophagy in metabolic syndrome

Metabolic syndrome is a collection of medical disorders, including obesity, insulin resistance, and dyslipidemia, which can lead to diabetes and cardiovascular disease. Under these energy-rich conditions, the Akt signaling pathway is activated. In turn, Akt phosphorylates and activates the mTOR kinase, a negative regulator of autophagy. Inhibition of mTOR has been linked to autophagy induction in metabolic syndrome. For instance, obesity was reported to inhibit autophagy in the liver. In addition to activation of Akt-mTOR signaling, obesity also induces the calcium dependent protease calpain, leading to cleavage and degradation of autophagy related protein 7 and ultimately inhibition of autophagy. Similarly, a recent study in Drosophila demonstrated that high fat diet induced obesity and cardiac dysfunction through activation of TOR signaling pathway and suppression of TOR signaling protected the heart against high fat diet induced cardiac dysfunction. Because TOR is a primary inhibitor of the autophagic pathway, it is reasonable to propose that high fat diet may inhibit autophagy in this model. However, Mellor et al. reported that up-regulation of autophagy was associated with decreased phosphorylation of Akt and S6 kinase, an mTOR downstream molecule, in a type 2 diabetic mouse model. In this animal model, twelve weeks of 60% fructose diet treatment induced systemic insulin resistance, as signified by impaired glucose tolerance and hyperglycemia. Concomitantly downstream signaling of the class I Phosphatidylinositol 3-kinases (PI3K) pathway was inactivated and the autophagic markers, lipidated LC3B (LC3BII/ LC3B-I) and p62, were up-regulated. The activation of myocardial autophagy was accompanied by elevated production of reactive oxygen species (ROS), fibrosis and cardiomyocyte loss (without indication of apoptosis induction). These results suggest that in insulin-resistant myocardium, suppression of Akt and S6 kinase as well as activation of autophagy have detrimental impact on cardiomyocyte viability in high fructose-induced diabetic mouse model. It is not yet clear how these contradictions may be explained. More investigations are warranted to determine how the PI3K-Akt signaling pathway can both promote and suppress autophagy in metabolic syndrome [14].

Targeting mitochondrial autophagy in diabetic cardiomyopathy

Significance: Diabetes is strongly associated with increased incidence of heart disease and mortality due to development of diabetic cardiomyopathy. Even in the absence of cardiovascular disease, cardiomyopathy frequently arises in diabetic patients. Current treatment options for cardiomyopathy in diabetic patients are the same as for nondiabetic patients and do not address the causes underlying the loss of contractility. Recent Advances: Although there are numerous distinctions between Type 1 and Type 2 diabetes, recent evidence suggests that the two disease states converge on mitochondria as an epicenter for cardiomyocyte damage. Critical Issues: Accumulation of dysfunctional mitochondria contributes to cardiac tissue injury in both acute and chronic conditions. Removal of damaged mitochondria by macroautophagy, termed "mitophagy," is critical for maintaining cardiomyocyte health and contractility both under normal conditions and during stress. However, very little is known about the involvement of mitophagy in the pathogenesis of diabetic cardiomyopathy. A growing interest in this topic has given rise to a wave of publications that aim at deciphering the status of autophagy and mitophagy in Type 1 and Type 2 diabetes [15].

Autophagy and Vitamin D

Vitamin D, vitamin D receptor, and macroautophagy in inflammation and infection

Vitamin D deficiency is a critical factor in the pathology of at least 17 varieties of cancer, as well as autoimmune diseases, diabetes, osteoarthritis, periodontal disease, and more. Vitamin D receptor (VDR) is a nuclear receptor that mediates most biological functions of vitamin D3, the active form of vitamin D. Activation of VDR signaling affects many processes, including calcium metabolism, apoptosis, immunity, and autophagy. Autophagy influences various aspects of disease progression, including stress adaptation, lifespan extension, development, immunity, and cancer. There is increasing concern regarding the use of vitamin D as a cheap and convenient supplement for disease prevention.

Vitamin D and VDR

There are two biologically relevant forms of vitamin D. One is ergocalciferol, or vitamin D2, and the other is cholecalciferol, or vitamin D3. The enzyme 25-hydroxyvitamin D-1alpha-hydroxylase, which catalyzes 25-hydroxyvitamin D3 into 1, 25(OH) 2D3, is critical to the production of the active form of vitamin D. After being taken up by target cells, vitamin D3 binds to its cognate receptor, VDR. VDR is a member of the nuclear receptor superfamily. In mammals, VDR is highly expressed in metabolic tissues, such as intestine, kidney, skin, and thyroid gland, and moderately expressed in nearly all tissues. Moreover, VDR is expressed in many malignant tissues. Active VDR binds to vitamin D response elements (VDREs) located in promoter regions of target genes, thereby controlling the transcription of these genes. VDR affects the transcription of at least 913 genes in human SCC25 cells (head and neck squamous cell carcinoma cell line). The impacted biological processes range from calcium metabolism to the expression of key antimicrobial peptides. Therefore, it is not surprising that vitamin D3/VDR signaling is involved in mineral and bone homeostasis, modulation of growth, cardiovascular processes, cancer prevention, and regulation of immune responses, including autophagy. Dysfunction of VDR and vitamin D3 deficiency can cause poor bone development and health, as well as increase the risk of many chronic diseases, including type 1 diabetes, rheumatoid arthritis, Crohn's disease, infectious diseases, and cancer.

Autophagy

Autophagy is a lysosome-mediated catabolic pathway that occurs ubiquitously in all eukaryotic cells. Depending on the route of delivery to the lysosome, autophagy is classified into three different types: macroautophagy (delivery of cytosolic contents to the lysosome by autophagosomes), microautophagy (inward invagination of the lysosomal membrane), and chaperone-mediated autophagy (direct translocation across the lysosomal membrane). We focus on macroautophagy, which is hereafter simply termed autophagy. The process of mammalian autophagy is divided into six principal steps: initiation or induction, nucleation, elongation, closure, maturation, and degradation or extrusion. Nucleation is the formation of the isolation membrane/phagophore. The nascent membranes are fused at their edges to form double-membrane vesicles, called autophagosomes. Elongation and closure lead to completion of the mature autophagosome. The autophagosome fuses with a lysosome to form an autolysosome, and then its content is degraded. More than 30 autophagy-related genes (ATG) regulate autophagy at the molecular level. The housekeeping function of autophagy is to maintain cellular energy levels and cell survival by recycling amino acids and fatty acids during periods of metabolic stress. Moreover, autophagy protects the cell by degrading damaged proteins and organelles as well as intracellular pathogens. The functions of autophagy include tumor suppression, antimicrobial defense, and inhibition of cardiac hypertrophy, anti-aging, and others. Remarkably, the functions of autophagy overlap with those of the vitamin D/VDR signaling.

Pathways involved in vitamin D3-associated autophagy

Autophagy can be induced by cellular stress, including starvation, hypoxia, biologic agents, and chemicals. Some studies have reported autophagy induced by vitamin D3 and its analogs in human myeloid leukemia cells, macrophages, breast cancer cells, and head and neck squamous cancer cells. The signaling pathways regulated by vitamin D3 include Bcl-2, beclin-1, mammalian target of rapamycin (mTOR), the class III phosphatidylinositol 3-kinase complex (PI3KC3), cathelicidin, calcium metabolism, and cyclin-dependent kinase. These pathways are critical in host defense and inflammatory responses. Hence, vitamin D3 and autophagy are associated with innate immunity, inflammatory bowel diseases, infection, and cancer.

Vitamin D3 increases free cytosolic calcium and decreases mTOR induction in autophagy: Vitamin D3 is a major regulator of calcium metabolism. Increased circulating vitamin D3 activates VDR, leading to increased intestinal calcium absorption. In excitable cells such as neurons, calcium is released from the sarcoplasmic or endoplasmic reticulum (ER) to activate calcium-dependent kinases and phosphatases, thereby regulating numerous cellular processes, including autophagy. ER calcium induces autophagy when stimulated by vitamin D3. This process is inhibited by mTOR, a negative regulator of macroautophagy, and induces massive accumulation of autophagosomes in a beclin-1- and ATG7-dependent manner since they are not fused with lysosomes. Vitamin D3 can down-regulate the expression of mTOR protein, thus inducing autophagy by inhibiting the mTORC1 complex. The Bcl-2 family also regulates autophagy. Vitamin D3 and vitamin D analogs significantly induced the expression of Bcl-2 in psoriasis. Vitamin D3 protected HL60 cells against apoptosis but down-regulated the expression of the Bcl-2 gene also vitamin D3-induced apoptosis of Y79 cells was accompanied by a reduction of Bcl-2 and increase of Bax protein. Bcl-2 inhibits autophagy by repressing calcium signals, depending on Bcl-2's location. Bcl-2 inhibits autophagy only when Bcl-2 resides in the ER, where it has been suggested to regulate cellular Ca^{2+} homeostasis.

Vitamin D3 regulates nucleation through beclin-1 and PI3KC3: Beclin-1 sits at the core of autophagy regulation. It is a key component of the PI3KC3 complex, which is important for the localization of autophagic proteins to a pre-autophagosomal structure. Beclin-1 is regulated by many factors, such as Bcl-2, NF-κB, vitamin D3, and vitamin D3 analogs. Inhibition of the VDR target gene cathelicidin significantly weakens vitamin D3-enhanced beclin-1 expression and vitamin D3-induced autophagy. However, the mechanism by which vitamin D3 increases beclin-1 expression remains unclear. Vitamin D3 can also increase beclin-1 expression through Bcl-2. Beclin-1 is a Bcl-2-homology-3 (BH3)-only protein. Bcl-2 binds directly to a BH3 domain in beclin-1, inhibiting beclin-1

and consequently autophagy. Silencing of endogenous Bcl-2 increases the level of starvation-induced autophagy, possibly due to the release of beclin-1 from the Bcl-2-beclin-1 complex, allowing a sufficient amount of beclin-1 to be recruited to bind to PI3KC3. Besides its effects on beclin-1, vitamin D3 signaling activates PI3K signaling pathway to induce autophagy. PI3K represents a family of kinases that phosphorylate the 3-hydroxyl group in phosphatidylinositol inositides .Vitamin D3 activates the PI3K pathway in THP-1 cells, enhances the expression of beclin-1, and induces the expression of PI3KC3 in leukemia cells.

Vitamin D3 increases lysosome function to promote maturation and degradation: Cathelicidin, a VDR downstream gene, is essential in autophagosome formation. Cathelicidin is recruited into autophagosomes through the Ca2+/calmodulin-dependent kinase (kinase-beta) and AMP-activated protein kinase signaling pathways in human monocytes treated with vitamin D3. VDR also regulates autophagy through p19INK4D. p19INK4D is a cyclin-dependent kinase inhibitor. Vitamin D3 induces the expression of p19INK4D in SCC25 cells, thus protecting cells from autophagy-induced death. It is clear that vitamin D3 signaling increases p19INK4D which in turn decreases autophagy and decreases VDR bound to the promoter of the p19INK4D gene. However, the mechanism of p19INK4D function in autophagy still remains largely unclear. Vitamin D analog EB1089 can increase the volume of the acidic compartment of lysosomes and the protease activity of lysosomes in a time-dependent manner starting before any apparent changes in cell morphology or DNA fragmentation are detectable. Therefore, vitamin D3 signaling can enhance autolysosome maturation and degradation.

Vitamin D/VDR regulation of inflammatory signaling in autophagy: Inflammation (inflammare in Latin, to set on fire) is the body's immediate response to damage to its tissues and cells by pathogens, noxious stimuli such as chemicals, or physical injury. Both autophagy and VDR signaling play critical roles in controlling inflammatory responses. Below we discuss in more depth the inflammatory signaling pathways associated with vitamin D and/or autophagy.

NF-κB affects nucleation through beclin-1

The nuclear factor-κB (NF-κB) family plays diverse roles in immunity, inflammation, and cancer. VDR down-regulates NF-κB activity. A NF-κB binding site is found in the promoter of the beclin-1 gene. Active NF-κB p65 up-regulates the expression of beclin-1 and stimulates autophagy in several cellular systems. Constitutively active IκB kinase (IKK) subunits stimulate autophagy. Inhibition or ablation of NF-κB p65 fails to suppress IKK-induced autophagy. At this point, it is clear that vitamin D3 signaling decreases autophagy through NF-κB. However, the effects of NF-κB on autophagy are inconsistent. In contrast to the stimulatory role of NF-κB in the regulation of autophagy, NF-κB has emerged as a negative regulator of autophagy, as induced by tumor necrosis factor, reactive oxygen species (ROS), and starvation in some cell lines. NF-κB inhibits starvation-dependent autophagy in the acute myeloid leukemia (AML) cell line U937. Prolonged NF-κB activation prevents E**.** coli-induced autophagy in macrophages by down-regulating the expression of Atg5 and beclin-1. Further research in various systems will be required to fully clarify the roles of NF-κB in autophagy.

Vitamin D3 may inhibit tumor necrosis factor-alpha (TNF-α) in autophagy: TNF-α is a pleiotropic inflammatory cytokine produced by activated immune cells as well as stromal cells. TNF-α significantly increases the expression of beclin-1 through the JNK pathway in human atherosclerotic vascular smooth cells. Vitamin D3 inhibits TNF-α in mycobacteria-infected macrophages and peripheral blood mononuclear cells from pulmonary tuberculosis patients. The vitamin D analog cholecalciferol reduces the circulating level of TNF-**α.** In addition, TNF-α significantly increases the expression of MAP1LC3 (ATG8) to induce autophagy. MAP1LC3 expression is induced via both the Akt and JNK pathways in human atherosclerotic vascular smooth cells. Therefore, vitamin D3 signaling may decrease TNF-**α**-induced autophagy.

NOD2 recruits ATG16 to regulate elongation: NOD2 is an intracellular pattern recognition receptor that recognizes muramyldipeptide (MDP), an integral component of bacterial cell walls. NOD2 is expressed in myelomonocytic cells, dendritic cells, and intestinal epithelial cells. NOD2 triggering by MDP induces autophagy in dendritic cells. This effect requires receptor-interacting serine-threonine kinase-2, ATG5, ATG7, and ATG16L. Vitamin D3 robustly stimulates the expression of the NOD2 gene and protein in primary human monocytic and epithelial cells. Moreover, NOD2 is known to trigger autophagy and eliminate intracellular bacteria through the recruitment of ATG16L1 to the site of bacterial entry. Therefore, vitamin D3 may increase vesicle elongation through the NOD2 pathway.

Autophagy via interferon-gamma (IFN)

IFN- is a cytokine produced by lymphocytes that has antiviral, immunoregulatory**,** and anti-tumor properties. Vitamin D3 inhibits IFN- **in n**aive CD62 ligand+CD4+ T cells and mycobacteria-infected peripheral blood mononuclear cells and macrophages. IFN- activates and increases lysosome activity in macrophages. It directly induces autophagy and the recruitment of autophagy proteins to the mycobacterial phagosome in macrophages. Autophagy induced by IFN- depends on ATG5. IFN- activation of macrophages also induces nitric oxide production, which in turn promotes autophagy through an autocrine positive-feedback loop. IFN- level increases when cells are under certain stresses, such as Salmonella infection. However, there is no direct evidence to show that vitamin D3 signaling may decrease autophagy through IFN.

Overall functions of vitamin D3 signaling in autophagy homeostasis

Vitamin D3 signaling affects autophagy at several levels, the outcome of which is two-sided. On one hand, vitamin D3 signaling increases the level of free cytosolic calcium and consequently decreases mTOR activity and induces

autophagy; vitamin D3 signaling also increases beclin-1 through several pathways, decreases the inhibition of Bcl-2, increases cathelicidin, and down-regulates NF-κB, which may decrease beclin-1 level. Vitamin D3 signaling can increase PI3KC3 protein, enhancing nucleation. To promote elongation, vitamin D3 signaling increases NOD2 level to recruit ATG16, increases lysosomal protease activity, and induces autophagosomes to fuse with lysosomes through cathelicidin. Vitamin D3 signaling regulates autophagy homeostasis. Vitamin D3 signaling may increase autophagy through the following factors: elevated cytosolic calcium; Beclin1, cathelicidin, and PI3KC3; and NOD2, lysosomal protease activity, and decreased NF-κB**.** On the other hand, vitamin D3 signaling may decrease autophagy through different mechanisms, especially under certain stresses. Vitamin D3 may decrease the level of NF-κB**,** TNF-α, or IFN- , thus decreasing autophagy. In addition, vitamin D3 increases the level of p19INK4D, which protects cells from autophagy-induced death.

Vitamin D, VDR, and macroautophagy in inflammation and infectious disease: Acute inflammation is considered a host defense strategy to remove the injurious stimuli. Inflammation plays a critical role in wound healing and infection resolution. Inflammation is not a synonym for infection. Infection is caused by an exogenous pathogen, such as bacteria, viruses, and parasites, whereas inflammation is one of the host responses to the pathogen. Although a successful inflammatory response is normally closely regulated by the body, inflammation could become pathologic and out of control. If the acute inflammation fails to eliminate the pathogen, the inflammatory process persists and acquires new characteristics. Chronic inflammation is a prolonged, dysregulated, and maladaptive response that involves active inflammation, tissue destruction, and attempts at tissue repair. Compelling evidence demonstrates that both vitamin D signaling and autophagy play a critical role in the pathogenesis of chronic inflammation and infection.

Vitamin D signaling and autophagy in inflammatory bowel diseases: Inflammatory bowel disease (IBD) is a dysregulated response of the immune system associated with intestinal tissues to the commensal microbiota in a genetically susceptible host. The major types of IBD are Crohn's disease (CD) and ulcerative colitis. The pathogenesis of IBD involves a complex interplay between genetic, microbial, immunological, and environmental factors. More than 30 genetic loci associated with IBD have been identified in genome-wide association studies. Autophagy-associated genes ATG16L1 and IRGM are confirmed susceptibility loci for CD. Variants in the NOD2 locus are associated with the strongest risk of developing CD. Mucosal inflammations in patients with IBD are accompanied by elevated levels of activated NF-κB, particularly p65. NOD2 and NF-κB play important roles in regulating autophagy. Paneth cells play an important role in intestinal innate immunity by means of secreting granule contents, including antimicrobial peptides and lysozyme. In experimental models, Paneth cells show notable abnormalities in the granule exocytosis pathway in ATG5- and ATG16L1- deficient mice. In human study, NOD2 mutations have been largely linked to ileal CD and have been associated with reduced expression of α-defensins HD-5 and HD-6 in isolates of ileal Paneth cells. Taken together, the data strongly implicate autophagy in the pathogenesis of IBD. Deficiency of vitamin D3 has been suggested as an important environmental factor for IBD. Vitamin D3 signaling regulates autophagy through several steps, which may affect the efficacy of treatments with vitamin D3 and its analogs on IBD. Vitamin D3 can increase NOD2 expression in human intestinal epithelial cells. In rabbits that were given a plant containing high levels of vitamin D3 for 15 or 30 days, time- and dose-dependent increases in the size and number of Paneth cells were found in the jejunum. In a pilot clinical study in IBD patients, reported a short-term beneficial effect on Crohn's disease activity after one-year administration of vitamin D3. However, there is no direct *in vivo* evidence of vitamin D3 signaling in the regulation of autophagy in IBD.

Vitamin D, autophagy, and infectious diseases: Some microorganisms have developed mechanisms to counteract or take control of the autophagic pathway as a survival strategy. Coxiella burnetii resides in a phagosome that interacts with autophagic vacuoles and then with lysosomes to generate a large replicative niche. This bacterially driven interaction with autophagosomes and its transit through the autophagic pathway favor Coxiella replication in the host cell. We speculate that vitamin D3 signaling may inhibit autophagy and kill the bacteria through cathelicidin. However, we found no published reports on the effects of vitamin D3 signaling and Coxiella burnetii. Cathelicidins are one of the major antimicrobial peptide families. In human, there is only one cathelicidin family member, human cationic antimicrobial protein (hCAP-18), which is cleaved to release LL37. LL-37 has shown a broad spectrum of activity against both Gram-negative and Gram-positive bacteria, various viruses, and fungi. In humans, cathelicidin contains activating VDREs in its promoter region, 507 bp upstream of its transcription initiation site. Activation of VDR results in the expression of cathelicidin at both the mRNA and protein levels in monocytes/macrophages. There is a long history of using vitamin D to treat mycobacterial infections. Vitamin D3's antagonism of M. tuberculosis involves antimicrobial peptides and autophagy. Vitamin D3-induced antimicrobial activity is completely inhibited in the presence of siRNA against cathelicidin. Hence, cathelicidin is essential for the induction of autophagy by vitamin D3 in bacterial infection [16].

Autophagy and Vitamin K

Vitamin K2 induces autophagy and apoptosis simultaneously in leukemia cells

Vitamin K2 (menaquinone-4: VK2) is a potent inducer for apoptosis in leukemia cells *in vitro*. HL-60bcl-2 cells, which are derived from a stable transfectant clone of the human bcl-2 gene into the HL-60 leukemia cell line, show 5-fold greater expression of the Bcl-2 protein compared with HL-60neo cells, a control clone transfected with vector alone.

VK2 induces apoptosis in HL-60neo cells, whereas HL-60bcl-2 cells are resistant to apoptosis induction by VK2 but show inhibition of cell growth along with an increase of cytoplasmic vacuoles during exposure to VK2. Electron microscopy revealed formation of autophagosomes and autolysosomes in HL-60bcl-2 cells after exposure to VK2. An increase of acid vesicular organelles (AVOs) detected by acridine orange staining for lysosomes as well as conversion of LC3B-I into LC3B-II by immunoblotting and an increased punctuated pattern of cytoplasmic LC3B by fluorescent immunostaining all supported induction of enhanced autophagy in response to VK2 in HL-60bcl-2 cells. However, during shorter exposure to VK2, the formation of autophagosomes was also prominent in HL-60neo cells although nuclear chromatin condensations and nuclear fragments were also observed at the same time. These findings indicated the mixed morphologic features of apoptosis and autophagy. Inhibition of autophagy by either addition of 3-methyladenine, siRNA for Atg7, or Tet-off Atg5 system all resulted in attenuation of VK2-incuded cell death, indicating autophagy-mediated cell death in response to VK2. These data demonstrate that autophagy and apoptosis can be simultaneously induced by VK2. However, autophagy becomes prominent when the cells are protected from rapid apoptotic death by a high expression level of Bcl-2 [17].

Vitamin K2-induced cell growth inhibition via autophagy formation in cholangiocellular carcinoma cell lines

Vitamin K2 (MK4) has antitumor effects on various types of cancer cell lines *in vitro*, and its efficacy has also been reported in clinical applications for patients with leukemia, myelodysplastic syndrome, and hepatocellular carcinoma (HCC). However, details of the mechanism of the antitumor effects of MK4 remain unclear. In the present study, we examined the antitumor effects of MK4 on cholangiocellular carcinoma (CCC) cell lines and its mechanism of action using the HL-60 leukemia cell line that exerts MK4-induced cell growth inhibition via apoptosis induction and cell cycle arrest as a control. MK4 exerted dose-dependent antitumor effects on all three types of CCC cell lines. However, apoptosis occurred in a smaller percentage of cells and there was less cell cycle arrest compared with other cancer cell lines studied previously, which suggested slight MK4-induced cell growth inhibition via apoptosis induction and cell cycle arrest. On the contrary, histopathological findings showed a large number of cells containing vacuoles in their cytoplasm, and electron microscopic findings showed a large number of cytoplasmic autophagosomes and autolysosomes. These findings suggested evidence of autophagy-related cell death. Fluorescence microscopy following acridine orange staining revealed an increase in the number of cytoplasmic acidic vesicular organelles characteristic of autophagy. Moreover, there were few cells forming autophagic vesicles in the control group, while the percentage of cells containing vacuoles in the MK4-treated group increased with the duration of culture. These results suggested that, unlike in leukemia, gastric cancer, HCC, and other cancer cells, the antitumor effects of MK4 on CCC cells are induced via autophagy formation [18].

The Role of Autophagy in Intracellular Pathogens Nutrient Acquisition

Food and reproduction are basic necessities for life. Intracellular pathogens infect host cells and are dependent on them for nutrients to propagate. While there is an abundance of food inside host cells, molecules are mostly sequestered in complex compounds or structures such as glycogen, lipid droplets, and proteins; forms that are not readily usable by microbial intruders. Therefore, simply gaining access to the interior of a host cell and avoiding potent innate antimicrobial host defenses is not sufficient to guarantee successful occupation and growth. Once inside, pathogens must either stimulate host cell import of metabolites or degrade intracellular storage molecules into compounds that can be transported and metabolized. There are multiple mechanisms by which intracellular pathogens accomplish this goal. For example, Mycobacterium tuberculosis encodes proteins to degrade host-derived lipids, such as cholesterol, for a carbon source. Pathogens can also take advantage of host signaling pathways to acquire nutrients. Both Brucella abortus and Salmonella enterica thrive on the increased glucose that is imported upon activation of various peroxisome proliferation-activated receptors (PPARs) in alternatively activated monocytes. Recently, several pathogens have been demonstrated to exploit host cell macroautophagy for nutrients. Autophagy is a critical mechanism that host cells use to increase nutrient availability when stressed. Since infection should exert a wide range of stresses on cells, it is not surprising that a diverse range of microbes have evolved strategies to extract the products of autophagy.

Autophagy is a highly conserved, multi-faceted eukaryotic process that maintains cellular homeostasis by degrading cytosolic material. Autophagy was noted as early as 1957 during the characterization of kidney cells by transmission electron microscopy (Clark, 1957, Deter and de Duve, 1967). In 1964, autophagy was identified as a mechanism to degrade cytosolic components and mitochondria under starvation conditions (Malkoff and Buetow, 1964). Since then, autophagy has been linked to a wide range of functions including antigen presentation through major histocompatibility complex II (MHC-II), unconventional secretion of inflammatory mediators, and cell viability. Autophagy is divided into several subsets based on the components being degraded. Bulk autophagy refers to non-specific cytoplasmic turnover while selective autophagy refers to autophagic degradation of specific structures. There are several distinct types of selective autophagy, which target specific cellular components such as mitochondria (mitophagy) or lipids (lipophagy). During infections, intracellular microbes are recognized, targeted, and degraded through a form of selective autophagy termed xenophagy. Although xenophagy is efficient at destroying microbes that enter the cytosol, intracellular pathogens have developed numerous evasion strategies to

avoid destruction by xenophagy, including the degradation or inhibition of autophagy components, camouflaging itself in host proteins, or blocking autophagosome maturation. Several pathogens that evade xenophagic killing have incorporated autophagy into their intracellular life cycle. These microbes exploit autophagy to sustain host cell viability, increase nutrient production, and/or for non-lytic exocytosis. Viruses also use autophagy or autophagy components for viral assembly and maturation.

Pathogens induce xenophagy

During infections, intracellular microbes are recognized, targeted, and degraded through a form of selective autophagy termed xenophagy. Inhibition of mTOR induces xenophagy in response to extracellular or phagocytosed microbes through Toll-like receptors (TLRs). TLRs recognize conserved microbial factors and initiate several anti-microbial processes, including xenophagy via Myd88 and Trif interacting with Beclin 1. Cell to cell signaling can also induce autophagy. Interferon gamma (IFN) activates autophagy through IRGM1 in human cells while CD40 ligation stimulates autophagy through PI3K and Rab7; priming cells to resist microbes. After phagocytosis, many pathogens escape the phagosome to replicate within the cytosol. The host cell mounts a xenophagic response to the membrane damage that occurs during phagosomal escape. Once microbes reach the cytosol, they can be targeted for xenophagy through immune surveillance or by causing cell stress. Several molecules, such as Nod-1 and Nod-2, identify microbial components within the cytosol to target microbes for xenophagy. Nod-1 and Nod-2 induce xenophagy and microbial antigen processing in response to bacterial peptidoglycan. Microbes also induce xenophagy through a number of cell stress mechanisms. B. abortus secreted TcpB induces endoplasmic reticulum stress via the unfolded protein response (UPR) pathway while Toxoplasma gondii increases intracellular calcium levels to induce autophagy. Lastly, xenophagy can also be directly induced by microbial proteins. For example, Shigella flexneri exported VirG polymerizes actin to propel the bacteria through the cytosol. ATG5 binds to VirG and initiates autophagosome formation without upstream autophagy signaling. However, S. flexneri also produces IcsB which blocks ATG5 from binding to VirG, thus inhibiting xenophagy. Likewise, the viral protein NS4B in Hepatitis C virus (HCV) induces autophagy by interacting with a Rab5/Beclin 1/VPS34 complex.

Pathogens have evolved complex xenophagy evasion mechanisms

Xenophagy is typically extremely effective at destroying microbes that enter the cytosol. For example, some serotypes of Group A Streptococcus (GAS) invade host cells, escape into the cytosol, and are then destroyed by xenophagy. Xenophagy effectively blocks these serotypes from using the cytosol as a replicative niche. To defend themselves, most intracellular pathogens have evolved mechanisms to either inhibit or evade xenophagy. Some GAS serotypes encode SpeB, which degrades the xenophagy adaptor proteins p62 and NRB1. GAS serotypes that are normally destroyed by xenophagy can be functionally complemented for xenophagy evasion and intracellular replication by expressing SpeB. To inhibit autophagy, pathogens frequently impair the function of xenophagy machinery. The RavZ protein secreted by Legionella pneumophilia inactivates LC3, effectively blocking autophagy in infected cells. Human Cytomegalovirus (HCMV), Herpes Simplex virus, and Kaposis sarcoma herpesvirus inactivate Beclin 1 to inhibit autophagy at specific points in their life cycle. Many viruses, such as Coxsackievirus, Hepatitis B virus, and HIV, inhibit autophagosome-lysosome fusion, functionally inhibiting xenophagy. The exact mechanism by which these viruses block autophagosome maturation is unknown, but many different RNA viruses encode proteins that interact with LC3, p62, NDP52, or NRB1. These proteins have several roles in xenophagy, but microbes may alter autophagosome maturation by manipulating these proteins.A few pathogens evade xenophagy without inhibiting autophagy. Listeria monocytogenes camouflages itself by binding to the host proteins ARP2/3, major vault protein (MVP), and ena/VASP. Many bacterial and eukaryotic pathogens modify phagosomes and are likely hidden from xenophagy targeting by remaining within a modified vacuole. M. tuberculosis and S. enterica typically reside in modified phagosomes but bacteria that disrupt the phagosomal membrane are rapidly destroyed by xenophagy. Vacuolar M. tuberculosis and T. gondii are degraded via autophagy when autophagy is stimulated by external sources, such as CD40 ligation or IFN- . Certain pathogens evade xenophagy by altering or destroying the components that target the microbes for degradation. S. enterica de-ubiquitinates aggregates with the effector protein SseL to prevent the aggregates from being degraded via autophagy. Likewise, B. pseudomallei encodes the de-ubiquitinase TssM which blocks several innate immune signals including the NF-kB and type 1 IFN pathways and has been proposed as a potential autophagy evasion mechanism. A few other cytosolic pathogens, such as Orientia tsutsugamuchi and F. tularensis, induce autophagy but the mechanisms of xenophagy evasion are not clear.

Pathogens harvest autophagy derived nutrients for replication

Intracellular microbes acquire nutrients from a range of sources, but generally rely on macromolecule degradation or nutrient import. Most basic nutrients within cells (amino acids, fatty acids, and carbohydrates) are incorporated into macromolecules (proteins, lipid droplets, and glycogen, respectively). In uninfected cells, these macromolecules are primarily degraded by autophagy to increase the amount of basic nutrients so the cell can build new structures. Thus, autophagy can increase the intracellular pool of nutrients that pathogens can access. Microbes can divert the nutrient by-products of autophagy toward microbial replication rather than for use by the cell. Dengue virus, F. tularensis, Anaplasma phagocytophilum, and T. gondii all induce autophagy, evade autophagic degradation, and harvest the autophagy derived nutrients for replication through

different mechanisms. Additionally, B. pseudomallei, Coxiella burnetii, and Leishmania amozonensis have impaired replication when autophagy is inhibited and nutrient acquisition has been implicated as a potential explanation for this phenotype. Dengue virus requires the degradation of lipid droplets via autophagy for optimal replication (Heaton and Randall, 2010). Dengue virus infections increase cellular levels of autophagy and the resulting autophagosomes form around and degrade lipid droplets. The triglycerides derived **from the lipid** droplets are catabolized via mitochondrial -oxidation, generating ATP. Thus, autophagy produces energy for the cell to indirectly enhance viral replication. In addition to energy production, Dengue virus modifies autophagosomes or amphisomes to form a replicative niche. Rather than being degraded through xenophagy, autophagy contributes to the maturation of infectious particles.

F. tularensis replicates in the cytosol of infected cells and induces an ATG5-independent, non-canonical form of autophagy. F. tularensis harvests amino acids from ATG5-independent autophagy for optimal intracellular replication. The amino acids are used for protein synthesis and are also metabolized as a major carbon source. F. tularensis bacteria are frequently adjacent to autophagosomes, indicating that F. tularensis is in the optimal physical location to compete with the host for autophagy derived nutrients. Although F. tularensis bacteria are frequently adjacent to autophagosomes, live bacteria are rarely degraded by xenophagy. O-antigen contributes to F. tularensis xenophagy evasion, but other effectors are also likely to be involved. A. phagocytophilum replicates in a vacuolar compartment and recruits autophagosomes directly to its replicative inclusions. A. phagocytophilum induces autophagy with the type IV secretion system (T4SS) effector Ats-1. Ats-1 binds to Beclin 1 and induces autophagosome nucleation directly rather than signaling through mTOR. Ats-1 induced autophagosomes localize with the inclusion membrane, suggesting that autophagosomes fuse with the inclusion body so that the bacteria can acquire the by-products of autophagic degradation. Inhibition of autophagy decreases A. phagocytophilum replication due to amino acid deficiency. Likewise, C. burnetti induces autophagy to enhance replication. C burnetti enters cells upon phagocytosis and modifies the phagosome to form a C. burnetti containing vacuoles (CCV). CCVs promiscuously fuse with other CCVs, endosomes and autophagosomes using the T4SS effector Cig2 (Newton et al., 2014; Winchell et al., 2014). When autophagy is impaired, CCVs do not fuse with one another and there is a severe replication defect (Gutierrez et al., 2005; Newton et al., 2014). The autophagosomes recruited to the CCV contain LC3, p62, and LAMP-1, suggesting that the autophagosomes that are recruited to CCVs have already fused with lysosomes. Since artificially enhancing autophagy further increases C. burnetti replication, the fusion of autophagosomes with the CCV has been postulated as a nutrient and membrane acquisition mechanism.T. gondii induces autophagy in infected host cells in a calcium dependent, mTOR independent manner. Inhibiting autophagy decreases T. gondii replication and parasite replication is rescued by supplementing with additional amino acids. Unlike its bacterial counterparts, fusion of T. gondii containing parasitophorous vacuoles (PVs) with autophagosomes leads to parasite destruction. T. gondii activates EGFR and AKT to inhibit PV-autophagosome fusion with EGF-MICs, primarily MIC3 and MIC6. Exploiting autophagy for nutrients is a recurrent theme in the pathogenesis of a diverse range of microbes. Several other microbes have enhanced replication when autophagy is induced and impaired intracellular replication when autophagy is inhibited, such as Chikungunya virus, B. pseudomallei and L. amazonensis. B. pseudomallei encodes the protein BPSS0180, which induces autophagy and is required for optimal intracellular replication. Similarly, L. amazonensis induces autophagy and has a replication defect when cells are deficient for autophagy. The role of autophagy in enhancing replication of these pathogens is unknown, but nutrient acquisition is a likely explanation for these phenotypes [19].

Role of Autophagy in Cancer

Autophagy is a cellular degradation pathway for the clearance of damaged or superfluous proteins and organelles. The recycling of these intracellular constituents also serves as an alternative energy source during periods of metabolic stress to maintain homeostasis and viability. In tumour cells with defects in apoptosis, autophagy allows prolonged survival. Paradoxically, autophagy defects are associated with increased tumorigenesis, but the mechanism behind this has not been determined. Recent evidence suggests that autophagy provides a protective function to limit tumour necrosis and inflammation, and to mitigate genome damage in tumour cells in response to metabolic stress. Autophagy is a cellular catabolic degradation response to starvation or stress whereby cellular proteins, organelles and cytoplasm are engulfed, digested and recycled to sustain cellular metabolism. Constitutive, basal autophagy also has an important homeostatic function, maintaining protein and organelle quality control, acting in parallel with the ubiquitin proteasome degradation pathway to prevent the accumulation of polyubiquitinated and aggregated proteins. Autophagy is also a pathway that is used for the elimination of pathogens and for the engulfment of apoptotic cells. However, the effect of these events on cancer is not known. Although most evidence supports a role for autophagy in sustaining cell survival, paradoxically, cell death resulting from progressive cellular consumption has been attributed to unrestrained autophagy. Complicating this situation further, cytotoxic events often induce autophagy, but whether this is a death mechanism or a futile effort at cellular preservation is often unclear. Another enigma has been the role of autophagy in tumour suppression; allelic loss of the essential autophagy gene beclin1 (BECN1, also known as ATG6) is found with high frequency in human breast, ovarian and prostate cancers, and autophagy-defective Becnl-heterozygous and autophagy-related 4C (Atg4C)-deficient mice are prone to tumors. Paradoxically,

most evidence supports a role for autophagy in maintaining tumour cell survival in response to metabolic stress *in vitro*, and in hypoxic tumor regions *in vivo*. Resolution of these paradoxes surrounding autophagy function has proved challenging.

The mechanisms that regulate the mutually opposed survival-supporting and death-promoting roles for autophagy are still far from resolution. The most plausible explanation is that catabolism through autophagy is predominantly survival-supporting, but that an imbalance in cell metabolism, where autophagic cellular consumption exceeds the cellular capacity for synthesis, promotes cell death. Although experimental evidence to support this is currently lacking, insight into the role of autophagy in tumour suppression is beginning to evolve. How loss of the pro-survival function of autophagy promotes tumorigenesis is partly explained by the stimulation of necrotic cell death and an inflammatory response in tumors with defects in autophagy and apoptosis. Preventing survival under starvation through autophagy, and diverting apoptosis-defective tumour cells to a necrotic cell fate, generates chronically necrotic tumors. This can corrupt a normal wound-healing response to support tumour growth, representing a possible means by which autophagy defects provide a non-cell-autonomous mechanism for stimulating tumorigenesis. In contrast to apoptosis, necrosis and cell lysis causes nuclear HMGB1 to be released from cells, and this and other events stimulate the innate immune response, the recruitment of inflammatory cells, cytokine production and nuclear factor-κB (NFκB) activation, which in some cases is linked to increased tumorigenesis. Indeed, blocking autophagy with constitutive activation of Akt in apoptosis-defective cells results in necrosis in response to metabolic stress *in vitro*, and in tumours *in vivo* this necrosis is coincident with NF-κB activation and promotion of tumorigenesis. How different cell-death processes interface with the immune system and tumour micro-environment to modulate tumour growth is far from clear and is an important area for future investigation.

How defective autophagy and compromised survival to stress can promote tumour progression despite reduced cellular fitness is suggested by the increased rate of cellular damage accumulation. In tumour cells in which cell-cycle checkpoints are inactivated, autophagy limits the accumulation of genome damage and suppresses the mutation rate. This supports the role for autophagy in protecting the genome in a cell-autonomous mechanism of tumour suppression. It is currently unclear how autophagy limits genome damage, but this could involve maintaining energy homeostasis or preventing the damaging effects of oxidative stress from defective organelle and unfolded protein accumulation. As we begin to define the role of tumour cell metabolism in response to stress, the rational ability to modulate the autophagy pathway in cancer therapy is emerging.

Apoptosis and metabolic stress

A common cellular response to metabolic stress is cell death by apoptosis, and in tumour cells in particular this apoptosis is crucial to suppressing tumorigenesis. The tumour-suppressive role for apoptosis is well known, originating with the realization that many tumours have apoptosis suppressed by various mechanisms, including over expression of the apoptosis inhibitor BCL2, and that preventing apoptotic cell death allows tumour cells to survive the stress of oncogene activation, uncontrolled proliferation and chemotherapy. Indeed, BCL2 antagonists have entered the clinic as part of the armamentarium to functionally restore the apoptotic pathway to resistant tumours. There is an entire family of multidomain (BCL2 homology regions or BH1-4) anti-apoptotic BCL2-homologous proteins that function to sequester the core pro-apoptotic regulators, multidomain BAX and BAK. Pro-apoptotic BH3-only proteins disrupt this BAX and BAK antagonism by BCL2-like proteins and some might directly activate BAX and BAK to propagate the death signal. Once activated, BAX and BAK oligomerize and permeabilize membranes, particularly the outer mitochondrial membrane, to release pro-apoptotic factors such as cytochrome c and SMAC/DIABLO. Cytochrome c is a cofactor for the apoptosome that activates the cysteine protease caspase 9, whereas SMAC/DIABLO inhibits the caspase antagonists, the inhibitor of apoptosis proteins (IAPs). Together, this results in effector caspase activation and widespread cellular protein substrate cleavage, causing cell death. Exactly how metabolic stress triggers apoptosis is not completely understood, but it is associated with the induction of the pro-apoptotic BH3-only protein PUMA, requires the BH3-only protein BIM, depends on either BAX or BAK, and is inhibited by BCL2. Once the apoptotic signal reaches BAX and BAK, mitochondrial membrane permeabilization, caspase activation and cell death occur in less than an hour and the process is extremely efficient and irreversible. Neighboring cells or professional phagocytes engulf apoptotic cells, preventing the activation of an innate inflammatory response. These attributes are the reasons why apoptosis defects are selected for in tumours and why restoring the apoptotic response is desirable for cancer therapy. Tumour cells with defects in apoptosis through either deficiency in BAX and BAK or gain of BCL2 or BCL-XL (also known as BCL2L1) function are highly resistant to metabolic stress and the properties of these 'undead cells' are revealing insights into the mechanisms regulating.

Metabolic stress has a major influence on tumours *in vivo*, either as a stimulator of apoptosis to limit tumour progression, or as a damaging insult in surviving apoptosis-defective tumour cells. Tumours are frequently subjected to metabolic stress, arising from the initial lack of a blood supply, vascular collapse in established tumours or therapeutic intervention. It is well known that tumour cells can be reliant on the inefficient process of glycolysis, rather than the more productive energy-generating process of oxidative phosphorylation, to support metabolism (the Warburg effect). As such, tumour cells are particularly vulnerable to metabolic stress, which is only exacerbated by the high energy demand of unrestrained cell growth and the potentially reduced capacity to access the nutrient

recycling activity provided by autophagy. How apoptosis-defective tumour cells adapt to metabolic stress and if or how this facilitates tumour progression has now been linked to autophagy. etabolic stress response.

Autophagy promotes survival

Evidence suggests that a constitutive, low level of basal autophagy in normal tissues provides an important homeostatic, housekeeping function. Targeted deletion of Atg5 or Atg7 in the brain causes the accumulation of polyubiquitylated protein aggregates and neuronal degeneration, supporting a role for autophagy in protein quality control. Furthermore, ATG5 is required for maintaining T-cell survival and proliferation37. Autophagy induction in response to stress and starvation also has a crucial role in normal cells. For example, Atg5-deficient mice fail to live through the neonatal survival period, during which tissues show signs of amino-acid depletion and metabolic insufficiency. These findings support a prosurvival role for autophagy in both normal tissues and in response to metabolic stress. In cancer cells, metabolic stress robustly induces autophagy, which is sustained when apoptosis is blocked. Importantly, autophagy is required for tumour cells to survive metabolic stress. Genetic inactivation of autophagy, either indirectly by constitutive activation of the phosphatidylinositol 3-kinase (PI3K) pathway or directly by allelic loss of Becnl or deficiency in Atg5, or by RNA interference (RNAi), prevents survival in response to metabolic deprivation even when apoptosis is inactivated. Presumably, in the absence of an external nutrient source the catabolic capacity of autophagy can sustain viability, but the role of autophagy in cell damage control and mitigation in response to stress may be equally important. Amino-acid starvation, glucose and oxygen deprivation, growth-factor withdrawal and cytotoxic cellular damage are among the many stimuli that potently induce autophagy. In the example of nutrient starvation, autophagy serves as a back-up energy reserve, whereas the autophagic response to cellular damage probably facilitates adaptation through the removal of damaged proteins and organelles.

Autophagy regulation

Autophagy is controlled mainly, but not exclusively, by the kinase mammalian target of rapamycin (mTOR; also known as FRAP1), which is a downstream component of the PI3K pathway. mTOR functions in part to suppress autophagy in response to nutrient and growth-factor availability. Conditions of starvation cause the de-repression of autophagy, which initiates isolation membrane or phagophore formation and the subsequent genesis of autophagosomes. Autophagosomes are double-membrane vesicles that sequester cytoplasm and organelles. The autophagy-regulated or Atg proteins are required for the activation of autophagy, the formation of autophagosomes, the sequestration of intracellular constituents, and the targeting and fusion of autophagosomes to lysosomes where the contents are degraded and recycled. For example, the serine/threonine protein kinase ATG1 (also known as ULK1) is a candidate for activation by mTOR de-repression that stimulates autophagy. ATG5 is required for autophagy and becomes covalently conjugated to the ubiquitin-like protein ATG12 by the ubiqutin-activating enzyme ATG7, but the specific role of this process in autophagy is not known. BECN1 is part of the class III PI3K VPS34 complex that is also required for autophagy. ATG8 (also known as MAP1LC3) is another ubiquitin-like protein that is cleaved, lipidated and becomes a component of the autophagosome membrane, and this membrane translocation event is commonly used to monitor autophagy.

The mechanics of phagophore and autophagosome formation and the recognition and capture of autophagosome cargo are presently unclear. Autophagy may be a nonspecific, bulk degradation process in some situations, and in others it may be specific for targeting mitochondria, catalase, peroxisomes, endoplasmic reticulum, and aggregation-prone proteins and protein aggregates for autophagy-mediated degradation. In the case of polyubiquitylated proteins, the multifunctional adaptor protein p62/SQSTM1 might facilitate the specific autophagosome-targeting process. If or how other aggregation-prone proteins, protein aggregates and organelles are recognized and targeted by the autophagy machinery is yet to be determined.

Autophagy in cell survival or death

In cancer cells, autophagy can, in some situations, increase apoptotic and caspase-independent57 cell death. However, autophagy has a more prominent role in sustaining cell viability in cancer cells with defects in apoptosis. Apoptosis-defective tumour cells have the remarkable ability to tolerate long-term metabolic stress, either by cytokine deprivation in dependent lymphoid cells or by oxygen (hypoxia) and glucose deprivation in epithelial cells. Although the absence of pro-apoptotic BAX and BAK or the gain of anti-apoptotic BCL2 or BCL-XL function adequately explains why cells fail to die when deprived, it is insufficient to explain how cell viability is maintained for weeks under the harsh metabolic stress conditions of nutrient deprivation *in vitro* and *in vivo*. It is now clear that surviving metabolic stress *in vitro* and in tumours *in vivo* is dependent on autophagy. The availability of cells with defects in apoptosis, with or without the capacity for autophagy (Becn1+/+ or Becn1 +/− and Atg5+/+, Atg5 +/− or Atg5−/−) has allowed a prolonged evaluation of the cellular response to metabolic stress, the survival from which is autophagy-dependent. Deprived epithelial and lymphoid cells using the autophagy survival function remain viable for weeks, during which time they undergo a dramatic decrease in cell size through progressive cellular consumption. Although BCL2 localized to the endoplasmic reticulum can interact with BECN1 and inhibit autophagy, in the functional context in which the normally predominantly mitochondrial BCL2 blocks apoptosis and confers tumorigenic growth, autophagy is efficiently induced in response to metabolic stress. It remains possible that the regulation of BECN1 by BCL2 is more subtle or context-dependent. It is clear from the analysis of time-lapse microscopy of apoptosis-defective immortal epithelial cells undergoing autophagy-mediated

survival as a result of starvation that this is a complex, poorly characterized and prolonged process. Initially, autophagy supports the continuation of cell proliferation under metabolic stress, which is consistent with a role for autophagy in the maintenance of homeostasis to support normal cell function during intermittent interruptions in nutrient availability. This 'maintenance phase' might also be crucial for sustaining ATP levels and cardiac function during the neonatal starvation period in mice.

Beyond two days of starvation cell division ceases, cells shrink markedly, motility is suppressed and cells aggregate into small clusters. These aggregated clusters of starved cells often show evidence of cells consuming other cells, or heterophagy, particularly when cells are in a confined space such as spheroid growth in Matrigel. Whether these cells are dead or alive at the time of consumption or if the persistence of these corpses reflects degradation failure due to defective autophagy remains to be determined. Furthermore, if or how this contributes to viability of the cell population is not determined. This 'preservation phase' is probably vital for controlling cellular consumption and mitigating protein and organelle damage while suppressing metabolism and energy use to prolong cell viability and enable recovery.

Autophagy enables stress recovery

Remarkably, autophagy affords cells a resilient capacity for regeneration, whereby restoration of nutrients results in an increase in cell size to that before starvation and resumption of cell proliferation. This recovery process is rapid and efficient, and dramatically impaired by defects in autophagy (Becn1+/− or Atg5−/−). Thus, autophagy-deficient cells not only fail to tolerate metabolic stress but are also defective in the recovery process. Mitigation of the damaging effects of stress, including damaged protein, DNA and organelle accumulation during this phase is probably crucial for enabling recovery. Following prolonged starvation and progressive autophagy, what defines the minimal cell that is capable of recovery and what events eventually lead to cellular demise are currently unknown. This capacity for durable, long-term survival of metabolic stress through autophagy might be vital for the survival of tumour cells that remain viable following treatment, for metastatic tumour cells and possibly for stem cells. In cells using autophagy to survive metabolic stress there is a gradual erosion of cell viability with time, but whether this is due to autophagic cell death or eventual cellular attrition through atrophy is not clear. Finally, the interaction of cells undergoing progressive autophagy, either dead or alive, with the immune system is unknown.

Autophagy and tumour dormancy

One of the most daunting clinical problems is the frequent re-emergence of tumours following treatment, often after prolonged dormancy59. How residual tumour cells cope with metabolic stress and remain viable yet dormant needs to be determined, as elimination of these tumour cells might be essential to achieving durable treatment responses. The survival of tumour cells through autophagy may be a key mechanism to enable long-term tumour-cell survival and eventual re-growth and relapse. Thus, autophagy may allow residual or metastasizing tumour cells to tolerate metabolic deprivation with the flexibility to recover once growth conditions are favorable. This dramatic capacity for recovery afforded by autophagy vaguely resembles the process of sporulation in microorganisms, and its suppression may be essential to achieve efficient cancer eradication.

Metabolic stress and necrosis

Inactivation of autophagy, either by allelic loss of Becn1, deficiency in Atg5, RNAi knockdown of expression of essential autophagy regulators, or constitutive activation of the PI3K pathway and mTOR-mediated inhibition of autophagy, prevents cells surviving metabolic stress. In apoptosis-defective cells this results in cell death by necrosis. What specifically triggers necrosis is unknown, but insufficient ATP production to maintain plasma-membrane integrity resulting in metabolic catastrophe and cell lysis is highly probable36. This is important because inhibition of autophagy is a means for sensitizing tumour cells to metabolic stress that is effective even in tumour cells with defects in apoptosis that would otherwise be difficult to eliminate. Although diverting apoptosis-defective tumour cells to a necrotic cell fate may not be a benign cell death, stimulation of acute necrotic cell death, if efficient enough, may be therapeutically useful. Indeed, the mechanism of cell death induction by alkylating agents involves poly (ADP-ribose) polymerase (PARP) activation and ATP consumption resulting in acute necrotic tumour cell death, which probably accounts for their success in the clinic62. Combining autophagy inhibitors with metabolic stress conditions might be a similarly effective means of promoting acute necrotic tumour cell death.

Necrotic cell death stimulates inflammation

Necrosis typically results from physical injury in which cell lysis and the release of intracellular contents, including HMGB1, activate the innate immune system and a wound-healing response. This recruitment of inflammatory cells provides cytokines, fostering cell growth to replace damaged tissue while removing cell debris. Once the tissue damage is repaired, the wound-healing response abates. Tumours, however, often show persistent, chronic necrosis and inflammation in a corrupted version of a wound-healing response. Necrotic tumours are associated with poor prognosis and the persistent inflammatory infiltration and cytokine production are thought to promote tumour growth. A high proportion of tumours have constitutive activation of the phosphatidylinositol-3 kinase pathway, which inhibits the induction of autophagy in response to metabolic stress, and many tumours also have defects in apoptosis. This generates a necrotic cell-death response to metabolic stress. A chronic necrotic response to persistent metabolic stress is created by rapid tumour growth and high metabolic demand that outpaces ATP production by glycolysis,

angiogenesis and nutrient availability. Thus, a necrotic cell fate is a common event in tumorigenesis, and evidence suggests that this alters the tumour- microenvironment interaction, although the mechanisms involved are poorly understood.

Autophagy and tumour suppression

The role for autophagy as a survival mechanism in normal cells and in tumour cells seems to contradict the observation that loss-of-function mutations in the autophagy pathway are associated with tumour progression. Furthermore, constitutive activation of the PI3K pathway is one of the most common events in human cancer, and the downstream kinase mTOR restricts autophagy induction in response to starvation64. How loss of this autophagy-mediated survival pathway promotes tumorigenesis was initially difficult to reconcile; however, two non-mutually exclusive possibilities have emerged. One explanation is that stimulation of necrotic cell death and inflammation caused by defects in apoptosis and autophagy provides a cell with non-autonomous means of tumour promotion through induction of a chronic wound-healing response. Another explanation is that proper management of metabolic stress through autophagy is required in tumour cells to suppress the accumulation of deleterious mutations, perhaps caused by the increased oxidative stress that can drive tumour progression. As such, overall cellular viability is compromised in tumour cells with defects in autophagy, but this initial disadvantage is overcome by an increased mutation rate resulting from failure of stress management in a cell-autonomous mechanism of tumour promotion. This is analogous to the mechanism by which defects in DNA repair cause sensitivity to DNA damage, yet the accelerated rate of mutation that results from deficient DNA repair confers an increased incidence of tumour formation.

Autophagy limits genome damage

Autophagy is required in stressed cells for maintaining protein and organelle quality control and energy homeostasis. A possible reconciliation of the pro-survival and tumour suppression functions of autophagy is that some aspect of the mismanagement of metabolic stress in autophagy-deficient tumour cells leads to genome damage and tumour progression. This could occur through protein, organelle and DNA damage, or insufficient ATP levels for essential cellular functions that are required to maintain genome integrity, such as mitosis and DNA replication and repair. This notion is supported by upregulation of the DNA damage response, evidence of DNA double-strand breaks, and aneuploidy in autophagy-defective immortal epithelial cells in association with increased tumorigenesis. This genome damage is manifested most obviously in cells with a defect in apoptosis, which would otherwise eliminate most of these damaged, abnormal cells, and is probably also facilitated by cell-cycle checkpoint inactivation (immortalization through RB1 and p53 loss). Increased genome damage resulting from an autophagy defect, however, does not require a defect in apoptosis, consistent with the tumour-prone state of Becn1-heterozygous mutant mice that have the apoptotic response intact. The origin of the increased DNA damage in autophagy-defective cells is not yet known. Malfunctioning organelles, accumulation of toxic protein aggregates, generation of reactive oxygen species and oxidative stress, and failure of energy homeostasis are all potential contributors to induction of genome damage when autophagy is defective.

Genetic instability and an enhanced mutation rate promote tumour-cell evolution and adaptation to drive progression and resistance to therapy. It is the rare, adapted, resistant tumour cells, which emerge typically following therapy, that are lethal. Autophagy deficient, immortal epithelial cells show an increased rate of gene amplification, the most common mechanism of oncogene activation in human tumours67, rendering this scenario plausible. Cancer is a disease where it is often the case that what grows back kills you, and what grows back is a mutated and more aggressive version of the original tumour generated by mutation, selection and genome instability. Despite the reduced cellular fitness caused by deficient autophagy, the poor survival but superior adaptation through an increased mutation rate might be the key advantage that promotes tumorigenesis. Interestingly, autophagy is associated with longevity, suggesting a role in the suppression of ageing phenotypes. As DNA damage accelerates both cancer and ageing, this supports a general role for autophagy in protecting cellular and genome fitness to prevent cancer and extend lifespan [20].

Autophagy and Apoptosis

Autophagy is a cell survival process which is related to breaking down and reusing cytoplasm components. Moreover, autophagy regulates cell death under certain conditions. Apoptosis has the characteristics of chromatin agglutination and the shrinking of nuclear and apoptosis body form. Even if the mechanisms of autophagy and apoptosis have differences, some proteins modulate both autophagy and apoptosis. Crosstalk between them exists. This review highlights recent advances in the interaction of autophagy and apoptosis and its importance in the development of cardiovascular diseases.

Molecular mechanisms of autophagy

Autophagy is a complex and evolutionarily conserved process. It is involved in the degrading of abnormal proteins and organelles. Autophagy is significant for maintaining cellular homeostasis under regular conditions, and is rapidly triggered by different stimuli such as nutrient starvation, hypoxia, oxidative stress, pathogen infection and endoplasmic reticulum stress. There are mainly three kinds of autophagy: (1) microautophagy, which directly sequesters and engulfs the cytoplasmic constituents via indentation inwards of the lysosome membrane; (2) chaperone-controlled autophagy, where cytosolic proteins with the KFERQ-like motif are recognized by chaperones, then unfold and translocate into the lysosome through the lysosomal-associated membrane protein type 2A; and (3) macroautophagy, which is characterized by formation of

the autophagosome (a double-membrane sequestering compartment) and fusing with the lysosome to deliver the cytoplasmic cargo. The process of macroautophagy (hereinafter referred to as autophagy) is as follows: induction, nucleation of the autophagosome precursor (phagophore), phagophore expansion and autophagosome maturation, fusing with the lysosome, and recycling of the degraded cargo. Autophagy Induction: The mammalian target of rapamycin (mTOR) integrates nutrient signals and cytokines from different pathways to inhibit autophagy and promote cell growth. Under stress or nutrient starvation conditions, mTOR is inhibited, which initiates autophagy by formation of the Unc-51-Like Kinase (ULK) complex including ULK, Autophagy-related Protein 13 (Atg13) and FAK-family Interacting Protein of 200 kDa (FIP200). Then the ULK complex phosphorylates Activating Molecule in Beclin-1-Regulated Autophagy (AMBRA1), which activates the phosphatidylinositol-3-kinase (PI3K) complex composed of Vacuolar Protein Sorting 15 (VPS15), VPS34, Beclin-1 and AMBRA1. During autophagy initiation, Beclin-1 is phosphorylated. Then Beclin-1 is separated from the dynein motor complexes, which are positively regulated by AMBRA1.

Autophagosome Formation: Once autophagy is induced, phagophore assembling is initiated by membrane nucleation. The membranes mostly originate from the mitochondria, endoplasmic reticulum, trans-Golgi network, late endosomes, and plasma membrane. Elongation and expansion of the phagophore membrane is an important stage in autophagosome formation. It is modulated by two inter-related systems of Atg12-Atg5-Atg16 and Atg8. In Atg12-Atg5-Atg16, Atg12 is initially triggered by the Atg7 in an ATP-dependent way. Then Atg12 is transferred to the E2-like conjugating enzyme Atg10 and forms the Atg12-Atg10 intermediate. Finally, Atg12 is covalently attached to Atg5. Further interplay between Atg5-Atg12 and the Atg16 homodimer leads to formation of the Atg12-Atg5-Atg16 complex. This complex locating to the phagophore is essential for autophagy. The second ubiquitin-like system induces the conjugation of phosphatidylethanolamine to Atg8/microtubule-associated protein 1 light chain 3 (LC3), which is subsequently processed by Atg4, Atg7 and Atg3. LC3-I is transformed into LC3-II. LC3-II is a special marker for the autophagosome. Autolysosome Formation and Recycling of the Degraded Cargo: Autolysosome formation originates from the transmitting and fusion of the autophagosome to lysosome. It is regulated by cytoskeleton and lysosomal membrane proteins. LAMP1/2 regulates autophagosome maturation. Gene mutation of LAMP2 causes Danon disease. Autophagosome accumulation and cardiomyocyte hypertrophy are characteristics of Danon disease. Once the autolysosome forms, the inner cargoes are degraded by lysosomal hydrolases. Catabolic products such as amino acids release into the cytoplasm for recycling and can be used as new resources.

Molecular mechanisms of apoptosis

Apoptosis is a process characterized by chromatin condensation, nuclear shrinkage and apoptosis body production. The apoptotic signaling cascade mainly includes two pathways, intrinsic and extrinsic, and it gets triggered by different mitochondrial stimuli or by molecules binding to the cell-membrane receptor. The intrinsic apoptosis signaling is induced by various stimuli, such as hypoxia, DNA damage, oxidative stress and deprivation of growth factor. It leads to mitochondrial membrane permeabilization. The integrity of mitochondria is regulated by different Bcl-2 superfamily members. They have the feature of the BH3 (Bcl-2 Homology) domain. Bcl-2 proteins are divided into two subcategories: pro-apoptotic and anti-apoptotic. Pro-apoptotic family members contain Bak, Bax, Bid, Bad, Noxa and PUMA. The anti-apoptotic family members include Bcl-2, Bcl-XL, Mcl-1, Bcl-W and A1/Bfl-1. The multi-domain pro-apoptotic proteins Bax and Bak are essential for inducing apoptosis. In response to stimulation of apoptosis, these proteins undergo conformational changes. It leads to their oligomerization on the outer membrane of mitochondria. Bcl-2 proteins block this pathway by interacting with Bax and Bak. It inhibits mitochondria permeabilization and cell death. After mitochondrial permeabilization, cytochrome c is released into the cytoplasm. Then cytochrome c binds to apoptotic protease-activating factor-1 (Apaf-1). It induces the conformational change and oligomerization of Apaf-1. This leads to the forming of a caspase-activating platform called the apoptosome. The apoptosome is comprised of Apaf-1, caspase-9 and cytochrome c. The apoptosome recruits, dimerizes and triggers caspase-9. Successively, it cleaves and induces caspase-3 and caspase-7. The last step of apoptosis is degrading DNA. The process is regulated by Endonuclease G. Endonuclease G is translocated from mitochondria to the nucleus and cleaves DNA.

The extrinsic apoptosis pathway is initiated through activating the death receptors. Death receptors bind to ligands and deliver apoptosis signaling. The cognate extracellular death ligands refer to soluble molecules of tumor necrosis factor (TNF). They are released as homotrimers and bind to the TNF-receptor (TNF-R). The TNF-R family is comprised of Fas/CD95, TNF-R1, TRAIL receptors-1 (TRAIL-R1), TRAIL-R2, DR-3 and DR-6. Ligand-binding makes the cell membrane receptors trimerize and activate [43]. TNF-Rs have a death domain (DD) that can recruit other DD-associated proteins. The DD-associated proteins include the Fas-associated protein with death domain (FADD) and TNF-R type 1-associated death domain protein (TRADD) these proteins bind to caspase-8 and - 10. Then the death-inducing signaling complex (DISC) is activated. DISC primarily activates caspase-8 and promotes the cell death outcome. Caspase-3 and -7 are cleaved after induction of caspase-8 and -10, which causes cell degradation.

Crosstalk between autophagy and apoptosis

Emerging evidence suggests interactions among the crucial proteins of autophagy and apoptosis, which underlie the molecular mechanism of the crosstalk between them.

Bcl-2/Beclin-1: The B-cell lymphoma 2 (Bcl-2) family proteins inhibit cytochrome c releasing from the mitochondria.

It plays a critical role in the apoptosis process. Beclin-1 is a component of the class III PI3K/Vps34 complex and is necessary for forming the autophagy vesicle. Bcl-2 binds to Beclin-1 and segregates Beclin-1 away from class III PI3K, leading to an inhibition of autophagic response. In contrast, mutations of either Beclin-1's domain or the BH3 receptor domain within Bcl-2 will disrupt the Bcl-2-Beclin-1 complex, which promotes autophagic activity. The interplay between Bcl-2 and Beclin-1 is essential to regulate the crosstalk between autophagy and apoptosis. Under the condition of sufficient nutrition, Beclin-1 and Bax/Bak bind to Bcl-2 or Bcl-XL. It inhibits activation of autophagy and apoptosis, respectively. Under conditions of starvation, autophagy is essential to guarantee cell survival. C-Jun N-terminal protein kinase 1 (JNK1) is activated and phosphorylates various residuum (Thr69, Ser70, and Ser87) of Bcl-2's regulatory loop. It leads to Bcl-2-Beclin-1 complex destruction, which induces autophagy. After autophagy activation, death-associated protein kinase (DAPK) phosphorylates the Thr119 of Beclin-1. It induces Beclin-1 separating from Bcl-2. Moreover, phosphorylating Beclin-1 on Thr119 decreases the Bcl-XL-Beclin-1 combining. It promotes autophagosome formation. The cytosolic translocation of high mobility group box 1 (HMGB1) is another factor promoting Bcl-2/Bcl-XL separation from Beclin-1. The intramolecular disulphide bridge (C23/45) of HMGB1 interacts with Beclin-1, which causes HMGB-1 to replace Bcl-2. Undergoing a long period of starvation cannot be relieved by autophagy. Phosphorylated Bcl-2 combines with Bax and inhibits apoptosis. The phosphorylated Bcl-2 protects cells against apoptosis through preserving the mitochondrial membrane completeness and preventing the pro-apoptosis proteins from releasing into the cytoplasm. However, in the situation of extreme starvation, JNK1 promotes hyper-phosphorylation of Bcl-2. It promotes Bcl-2 separating from Bax. Then it induces apoptosis via caspase-3-dependent pathways and, subsequently, a safe cell death.

Atgs: Autophagy-related proteins (Atgs) involved in various stages of autophagy have also been shown to regulate the apoptotic pathway. Gene mutation or inhibition of these specific Atgs may affect the apoptosis process. Atg3 is a non-canonical ubiquitination E2 enzyme regulating the conjugation of ubiquitin-like Atg8 to phosphatidylethanolamine in the autophagy process. In addition, recent studies have shown that Atg12 covalently conjugates to Atg3. The Atg3-Atg12 complex localizes to the mitochondrial outer membrane, regulating mitochondrial homeostasis and cell death. Atg12 conjugation to Atg3 sensitizes cells to mitochondria-mediated apoptosis. However, it has no effect on death receptor-mediated apoptosis. Disturbing the complex formation, selective mitochondrial autophagy (also called mitophagy) is reduced significantly, but it has no effect upon non-selective autophagy.

Atg4 is a cysteine protease cleaving the C-terminus of LC3, which has an effect on the covalent attachment of newly synthesized Atg8 to PE and on the delipidation of Atg8 at the lysosomal fusion stage. Atg4D, a human Atg4 member, is cleaved by caspase3 and generates two fragments in the apoptosis cell. The N-terminal fragment of Atg4D cleaves and delipidates the Atg8 paralogue -aminobutyric acid receptor-associated protein-like 1 (GABARAP-L1), which leads to the decrease of autophagosome formation. The C-terminal fragment with a putative BH3 domain is recruited to the mitochondrial matrix, promoting the mitochondria-mediated apoptosis. Covalent conjugation of the autophagy-related proteins Atg5 and Atg12 involved in an ubiquitylation-like process is essential to autophagosome formation. Atg5 and Atg12 are, therefore, integral parts of the autophagic machinery and are required for the induction of autophagy. Hence, Atg5 and Atg12 are absolutely necessary for autophagic activity. Interestingly, it has been found that Atg5 and Atg12 also participate in apoptosis initiation in response to various stress signals. Moreover, non-conjugated forms of Atg5 and Atg12 have an effect on the induction of apoptosis, which indicates that their effect on apoptosis is likely to be independent of their effect on autophagy. Atg5 has a double role in regulating autophagy and apoptosis. One study reported that over-expression of Atg5 made the tumor cells sensitive to chemotherapy, while silencing the Atg5 gene with short interfering RNA made the cells partially resistant to chemotherapy. This study showed that, during apoptosis, Atg5 was cleared by calpains, producing an amino-terminal cleavage product. Truncated Atg5 moved from the cytoplasm to mitochondria. Then it interacted with Bcl-XL and promoted cytochrome c release and caspase activation. Atg5 cleavage was found independent of the apoptotic stimulus and cell type. It was indicated that calpain induction and Atg5 cleavage were universal phenomena in apoptotic cells. On the contrary, without Atg5 in mitochondria, autophagy takes place. Atg12 has a dual function, participating in autophagy and apoptosis, which connects both of the processes. Non-conjugated Atg12 can combine with and inhibit Mcl-1 and Bcl-2 by a BH3-like motif, which positively regulates mitochondrial apoptosis. In the apoptosis cell, knockout of Atg12 inhibits Bax induction and cytochrome c release. On the contrary, aberrant expression of Atg12 represses the anti-apoptotic activity of Mcl-1. In addition, a recent study demonstrated that free Atg12 was unstable. It could be broken down in a proteasome-dependent way. Atg12 could be directly ubiquitinated, which promotes the proteasomal degradation. Free Atg12 could cause proteasome inhibitor-regulated apoptosis, indicating proteasome inhibitors might be potential anticancer agents in clinical practice.

Caspases

Caspases are both the initiators and effectors participating in apoptotic cascades. Recently, it has been found that caspases participate in regulating the crosstalk between autophagy and apoptosis. Various pro-apoptosis pathways can induce caspases to trigger apoptosis. Moreover, activated caspases could cleave and break down the critical autophagic proteins (such as Beclin-1, p62, Atg3, Atg4D, Atg5, Atg7, and AMBRA1). It leads to an inactivation

of their autophagic function. Surprisingly, some pro-autophagic proteins can even be transformed into pro-apoptotic proteins to initiate apoptosis cell death after being cleaved by caspases. In addition, autophagy can have an effect on apoptotic cascades through modulating the caspases. Caspase-2 is an important regulator of cascades in a context-dependent way. Recent research reported that mice neurons in the absence of caspase-2 cannot execute apoptosis, while autophagy is activated at an early stage. It causes a response to rotenone-regulated mitochondrial oxidative stress. It has also been found that, in mouse embryonic fibroblasts, a lack of caspase-2 contributes to an enhanced autophagy. Caspase-3 is a predominant effector caspase in apoptosis. However, accumulating studies have shown that caspase-3 is essential to autophagic activity. A study reported that, during staurosporine-induced apoptosis, caspase-3 could cleave Beclin-1 on 124 and 149. It inhibited autophagy and activated apoptosis in HeLa cells. Another study found that caspase-3, together with other caspases, cleaved Beclin-1 in the apoptosis process. It regulated by IL-3 deprivation in culture medium, blocking autophagic activity and promoting the pro-apoptotic stimulus. The Beclin-1 C-terminal fragment localized at the mitochondria. Subsequently, it sensitized the cell to apoptosis.

Caspase-6 is also an effector caspase in apoptosis. It has been demonstrated that caspase-6 cleaves p62 and Atg3, which suggests its importance in mediating autophagy. Moreover, when melanoma cell lines suffer arginine withdrawal, TRAIL-induced caspase-6 activation disrupts autophagy by cleavage of two crucial autophagy proteins, Atg5 and Beclin-1. Caspase-8 is an essential trigger involved in death receptor-induced apoptosis. The increasing evidence indicates that caspase-8 also participates in regulating autophagy. During the death receptor-triggered apoptosis, caspase-8 cleaves Atg3, targeting the conserved LETD sequence (Atg3 amino acids 166-169), which inactivates the pro-autophagic activity. In addition, caspase-8 could prevent T cells from hyperactive autophagy. Caspase-9 is also a key triggering caspase participating in intrinsic apoptosis. It has been reported that caspase-9 interacts with Atg7 at the C-terminal region. It promotes LC3-II formation and autophagy activity. The interplay between caspase-9 and Atg7 hinders the recruiting and processing of caspase-9 in apoptosomes, inhibiting caspase-9 activation and apoptosis. Moreover, in breast cancer MCF-7 cells, suppression of caspase-9 can block the autophagic flux and induce the cell death by inhibiting cytoprotective autophagy.

P53

p53, a signal transduction integrator, can be induced by diverse abnormal conditions, including hypoxia, DNA damage, nutrient stress, and ischemia-reperfusion. p53 has an effect on regulating apoptosis both through the intrinsic and extrinsic pathways. In the nucleus, p53 promotes the pro-apoptotic proteins (such as Bax, Bid, PUMA, and Noxa) and inhibits Bcl-2 expression, which triggers the intrinsic apoptotic pathway. In the cytoplasm, p53 promotes the TRAIL receptor and Fas receptor, causing the initiation of the extrinsic apoptotic pathway. In addition, p53 can activate Apaf-1 of the apoptosome. Recently, an increasing number of studies have indicated that p53 is also involved in the regulation of autophagy. It is reported that genotoxic stress induces autophagy through transcriptional activation of a direct p53 target gene, damage-regulated autophagy modulator (DRAM), whose signaling cascade promotes autolysosome formation. DRAM is essential for the network regulating p53-regulated apoptosis and autophagy [79]. Another study showed cytoplasmic p53 suppressed autophagy by inactivating AMP activated protein kinase (AMPK) and subsequently activating mTOR signaling. It also has been investigated that, under the nutrient deprivation condition, p53 post- transcriptionally down regulates LC3, which controls the autophagic flux and prevents the cells from **"**autophagic burst**"**. Moreover, inhibition of p53 triggers autophagy mostly in the G1 phase and less in S phase, but never in the G2/M phases. It is strictly cell cycle-dependent.

FLIP

FADD-like IL-**1** -converting enzyme-inhibitory protein (FLIP) is an anti-apoptotic protein, suppressing death receptor-mediated apoptosis. Recently, it has been shown that FLIP competes with LC3 for the binding of Atg3 and inhibits LC3 lipidation, which suppresses autophagy. On the contrary, once the autophagy is triggered, the interaction of FLIP and Atg3 is significantly decreased.

Mitoptosis

Mitoptosis is an apoptotic-like process inside mitochondria. It occurs mainly as an outcome of mitochondrial outer membrane permeabilization (MOMP) and potential loss. It has been demonstrated that dysfunction of the mitochondria and production of ROS are essential for inducing mitoptosis. It also has been reported that following the Bax/Bak-regulated MOMP, DDP/TIMM8a, a mitochondrial intermembrane space (IMS) protein, is released into the cytoplasm where it binds to and promotes the mitochondrial redistribution of Drp1. The interplay promotes Drp1-regulated fission of mitochondria and, subsequently, mitoptosis. An increasing number of studies indicate that disruption of mitochondria can cause promotion of autophagy. Indeed, a study reported activation of mitoptosis and the subsequent destruction of ATP was followed by the induction of autophagy to maintain the energy. Another study found that clearing away abnormal mitochondria may be either be done through autophagosome formation via selective mitochondrial autophagy (mitophagy) or through the formation of mitoptotic bodies. Then they are released into the extracellular space via atypical exocytosis.

Mitophagy

Mitophagy is the process of recognizing and removing abnormal mitochondria via autophagy-regulated degradation. Recent research has demonstrated that mitochondrial dynamics are essential to mitophagy. Mitochondrial fission is regulated by the GTPase dynamin-

related protein 1 (Drp1). Mitochondrial fusion includes three GTPases: optic atrophy 1 (OPA1) induces inner membrane fusion and mitofusins 1 and 2 (Mfn1 and Mfn2) regulate outer membrane fusion. Mitochondria are divided into depolarized and polarized mitochondria after fission. Depolarized daughter mitochondria are targeted by mitophagy, while polarized mitochondria undergo fusion. Interestingly, accumulating evidence suggests that mitophagy undergoes extensive crosstalk with apoptosis pathways. Mitochondrial dynamics are crucial for the crosstalk between mitophagy and apoptosis. A study reported that Parkin underwent extensive crosstalk with apoptosis pathways. Mitochondrial translocation of Parkin was inhibited by pro-survival Bcl-2 proteins. It was triggered by BH3-only proteins under conditions of inhibited caspase activity. Undergoing this condition for a long time, Parkin could promote apoptosis by degrading anti-apoptosis Mcl-1. Another study also found that the mitochondrial deubiquitinase USP30 opposed parkin-regulated mitophagy. Knockdown of USP30 could induce the mitochondrial apoptosis pathway. These findings indicated that USP30 would make mitochondria induce mitophagy rather than apoptosis. Furthermore, pre-promotion of Bnip3-mediated mitophagy by constitutively activating the Bnip3 receptor ahead of tumor necrosis factor (TNF) treatment inhibited effector caspase activation significantly. It suggested that the activation of mitophagy or delayed induction of membrane permeabilization inhibited apoptosis. However, diverse feedback between individual mitophagy programs and both pro-survival and pro-death apoptosis pathways occurred at different time scales and underwent crosstalk.

The relationship between autophagy and apoptosis in cardiac diseases

In physiological conditions, autophagy and apoptosis play essential roles in cardiac health and integrity. The structure and function of cardiac myocytes is closely related to autophagic flux. Cardiac myocytes retain a limited ability to enter the cell cycle again. It leads to a limited capacity for regeneration in the adult heart. As a consequence, there exists a continuous process of cell renovation. It includes removal and replacement of damaged tissue. In addition, autophagy is necessary for continual heart contraction. It is also critical for large cytoplasmic calcium transients without disturbing cardiac function. During heart development, apoptosis participates in the development of the embryonic outflow tract, cardiac valves, conducting system, and coronary vasculature. In pathological conditions, the interplay between autophagy and apoptosis are closely related to some cardiac diseases involving ischemic heart disease, pressure overload-induced cardiac disease and diabetic cardiomyopathy.

Ischemic heart disease

Programmed cell death of cardiac myocytes takes place following ischemia/reperfusion (I/R), leading to cardiac dysfunction. It has been proposed that I/R causes cell death via apoptosis and necrosis. Currently, it was reported that autophagy was an essential regulator of programmed cell death, either inhibiting or promoting apoptosis, or acting as a programmed cell death distinct from apoptosis. It is generally believed that promotion of autophagy is protective during myocardium ischemia. The myocardial ischemia swine models were induced by one, three, or six episodes of 90 min of left anterior descending coronary stenosis (30% decrease in baseline coronary flow) followed by reperfusion every 12 h, while the non-ischemic regions were the control. This study indicated that a chronically ischemic myocardium activated autophagy and inhibited apoptosis, which could limit the deleterious effects of chronic ischemia and protect against further ischemia. It also has been shown that autophagy is activated by ischemia and reperfusion in the mouse heart *in vivo*. Under the condition of prolonged ischemia, inhibition of autophagy was accompanied by the expansion of myocardial infarction size, which suggested that the activation of autophagy protected the cardiac cells during ischemia. Moreover, it was found that ischemia induced autophagy through the AMPK-dependent signaling pathway, while reperfusion stimulated autophagy by the upregulation of Beclin-1 and BNIP3, but without AMPK activation. In cardiac myocytes, the reduction of Beclin-1 expression by RNA interference inhibited I/R-induced autophagy, which involves enhanced cell survival. The inhibition of NF-κB suppressed Beclin-1 expression and autophagy. It reduced the extent of the cardiac area at risk for ischemia. It also reported that mitochondrial c-Jun N-terminal kinase (JNK) activation induced autophagy and apoptosis, aggravating myocardial I/R injury. Insulin selectively inhibited mitochondrial JNK activation, protecting cardiocytes against I/R injury. Recently, one study was aimed at investigating the effects of berberine, a natural extract from Rhizoma coptidis, on ischemia/reperfusion-induced excessive autophagy. Autophagy was induced both in H9c2 myocardial cells under the hypoxia/reoxygenation (H/R) condition, and in mouse hearts exposed to I/R. The results showed that berberine treatment significantly strengthened the viability of H/R-induced cells, decreased the I/R-induced myocardial infarct size, and improved the heart function. The therapeutic effect of berberine is associated with down regulating the expression of autophagy-associated proteins such as SIRT1, BNIP3, and Beclin-1, and suppressing autophagy activity. Furthermore, the levels of p-AMPK and p-mTORC2 (Ser2481) in H9c2 cardiomyocytes exposed to H/R were down regulated by berberine. One study suggested that vitamin D receptor was a potential endogenous self-defensive and cardio protective receptor protecting against myocardial I/R injury via inhibiting autophagy dysfunction-regulated cell death and apoptosis. Another study indicated that sphingosylphosphorylcholine protected cardiomyocytes against ischemic apoptosis by promoting lipid raft/PTEN/Akt1/mTOR-regulated autophagy. In addition, a recent work demonstrated Mst1, a crucial protein of Hippo signaling, improved the heart disorder in mice suffering myocardial infarction via suppressing autophagy. The mechanism was that Mst1 phosphorylated the Thr108 residue in the BH3 domain of Beclin1. It enhanced the interplay between Beclin1 and Bcl-2 and/or Bcl-xL, and stabilized the Beclin1

homodimer. It also suppressed the phosphatidylinositide 3-kinase activity of the Atg14L-Beclin1-Vps34 complex and subsequently inhibited autophagy. Mst1-mediated sequestration of Bcl-2 and Bcl-xL by Beclin1 activated Bax and promoted apoptosis. Taken together, autophagy is activated during myocardial ischemia and further enhanced by reperfusion. Autophagy is protective during the ischemic phase, while it is harmful in reperfusion. It is supposed that activation of regular autophagy and inhibition of abnormal autophagy and apoptosis can rescue myocardial cells against death during ischemia/reperfusion.

Pressure overload-induced cardiac disease

Although accumulating research has paid close attention to the role of autophagy and apoptosis in pressure overload-induced cardiac disease, it is still unclear whether they play positive or negative roles in cardiac disease. A study reported that in adult mice, knockout of cardiac-specific Atg5 led to cardiac hypertrophy. It also caused left ventricular expansion one week after treatment with thoracic transverse aortic constriction (TAC). These results suggested that under baseline conditions, regular autophagy was a homeostatic mechanism for maintaining the structure and function of the heart. Autophagy activation was an adaptable reaction for preventing hemodynamic stress in heart failure. Another study found that berberine could effectively attenuate cardiomyocyte apoptosis and left ventricular remodeling through an autophagy-dependent mechanism in rat cardiac hypertrophy models induced by TAC. The potential mechanism was related to inducing autophagy by the suppression of mTOR activity and its upstream p38 and extracellular signal-regulated kinase (ERK1/2) mitogen-activated protein kinase (MAPK) signaling pathways. In contrast, some research suggests that autophagy has a detrimental effect on pressure overload-induced cardiac disease. A study reported that pressure overload induced by aortic banding significantly enhanced cardiac autophagy and led to heart failure. Pressure overload-induced autophagy reached the peak at 48 h. It kept rising for at least three weeks. Heterozygous disruption of Beclin-1 gene coding inhibited cardiomyocyte autophagy and alleviated pathological remodeling induced by TAC. On the contrary, Beclin-1 over-expression increased autophagy and pathological remodeling. Nevertheless, it was ambiguous if apoptosis participated in later stages of pathological remodeling. Another research found that in the renal artery stenosis-induced experimental hypertensive swine model, activation of autophagy and apoptosis participated in left ventricular hypertrophy and pathological remodeling. It indicated that autophagy could portend the level of cardiac hypertrophy.

One study also showed that activated transcription factor 3 (ATF3) protected against pressure overload-induced heart failure. The mechanism was bound to the ATF/cAMP response element of the Beclin-1 promoter and inhibited autophagic activity by inhibition of the Beclin-1-dependent pathways. In addition, the crosstalk between apoptosis and autophagy regulates proliferation and death of cells in pulmonary hypertension pathogenesis, especially in pulmonary vascular remodeling involving endothelial cells and smooth muscle cells [21].

Autophagy and Aging

Autophagy, a highly conserved mechanism of quality control inside cells, is essential for the maintenance of cellular homeostasis and for the orchestration of an efficient cellular response to stress. The decrease in autophagic activity observed in almost all cells and tissues as organisms age was proposed to contribute to different aspects of the aging phenotype and to the aggravation of detrimental age-related diseases. The recent advances in our understanding of the molecular mechanisms underlying autophagy and the identification of the subset of genes involved in this process has enabled the use of genetic manipulations to start testing this hypothesis.

Cellular quality control, autophagy and aging

All cells rely on surveillance mechanisms, chaperones and proteolytic systems to control the quality of their proteins and organelles and to guarantee that any malfunctioning or damaged intracellular components are repaired or eliminated. Molecular chaperones interact with unfolded or misfolded proteins and assist in their folding. However, if the extent of protein damage is too great, or the cellular conditions are not adequate for re-folding, the same molecular chaperones often deliver proteins for degradation. Two proteolytic systems contribute to cellular clearance: the ubiquitin-proteasome and the lysosomal systems. Chaperone malfunctions or alterations in the components of the proteolytic systems result in intracellular accumulation of damaged proteins and organelles and underlie the basis of different human pathologies. Accumulation of damage is also characteristic of tissues in all organisms as they age and has been proposed to be responsible for their functional loss in aging. Changes with age in both the ubiquitin-proteasome and the lysosomal system have been described, but only recently has the contribution of these changes to the aging phenotype started to be elucidated. The main reason for the recent advances has been the improved molecular characterization of the different cellular degradative pathways. The identification of the genes encoding effectors and regulators of intracellular clearance has now enabled direct analysis of the consequences of down regulation of their activity to levels similar to those observed in old organisms. Likewise, genetic manipulations to prevent the age-related functional decline of some of these systems have confirmed the contribution of the accumulation of intracellular damage to their functional failure in aging. Here, I review the contribution of the lysosomal system to the maintenance of cellular homeostasis - through autophagy- and the recent findings linking the autophagic system to life-span extension and to different aspects of cellular and organismal aging.

Autophagy: the comeback of an old pathway

Lysosomes are organelles fully devoted to degrading diverse macromolecules both from the extracellular environment and from inside the cells. Lysosomes contain the highest

cellular concentration of hydrolases (i.e. proteases, lipases, glycases and nucleotidases) in their lumen, in addition to permeases in their membrane for recycling the essential building blocks of the degraded products (e.g. amino acids, fatty acids and cholesterol, sugars, etc.) to the cytosol. In this respect, lysosomes are real recycling compartments in which cellular structures are broken into their individual components, which can then be reused for synthesis of new cellular structures. Lysosomes degrade both intact (functional) proteins, to guarantee continuous renewal of the cellular proteome, and damaged proteins that are no longer functional, to avoid their accumulation inside cells. Lysosomes not only degrade soluble individual proteins but also particulate structures and complete organelles. This feature makes the lysosomal system particularly relevant under conditions when damaged proteins start to organize into irreversible oligomers and aggregates. The contribution of the lysosomal system to catabolism and intracellular clearance (autophagy) has been known for more than half a century, since the description of this organelle by deDuve . However, until recently, the understanding of the pathophysiology of autophagy advanced at a very slow pace compared to that of other quality control mechanisms. The most important propellers of the current advances in the autophagy field were three simultaneous yeast genetic studies initiated 10 years ago, which identified the first autophagy related genes (ATG). The numbers of ATG genes have escalated to include >30 and the majority are conserved throughout evolution. The possibility of genetically manipulating autophagy - through knockouts, knock-downs and over expression of the ATG genes - has permitted, for the first time, investigation of the cellular consequences of changes in the activity of this pathway and a link between autophagic malfunction and different human diseases.

Autophagy and aging: before the genetic dissection

A decrease in proteolytic activity has been considered responsible, at least in part, for the accumulation of damaged cellular components in almost all tissues of aging organisms. Indeed, age-dependent alterations in the lysosomal system and declined autophagic activity were described long before the molecular basis for this process was fully understood. Most of the early functional studies were performed in the liver, as this was also the organ in which the most extensive characterization of the different autophagic pathways was available. However, the morphological features of the aging lysosomal system (e.g. expansion of lysosomal compartments, accumulation of autophagic vacuoles and deposition of undigested material inside lysosomes in the form of an auto fluorescent pigment termed lipofuscin) were also described in non-dividing cells of many other tissues (e.g. brain, heart, muscle and kidney). Failure of the quality control mechanisms are particularly detrimental in post-mitotic cells, in which distribution of damaged components to daughter cells is not possible because their cells no longer undergo cell division.

Measurement of autophagic activity (the rate of degradation of long-lived proteins) in rodent livers revealed an age-dependent decrease in lysosomal-mediated degradation that correlated with an increase in damaged proteins in this organ. The reasons for this reduced macro autophagic activity with age, at least in the liver, seem to be twofold: a defect in the clearance of autophagic vacuoles and problems in the hormonal regulation of this type of autophagy. The half-life of newly formed autophagosomes most likely increases because of the inability of the secondary lysosomes to fuse and/or degrade the autophagosome cargo. Failure of the lysosomal hydrolases, as a result of the gradual increase of lipofuscin in lysosomes and of primary damage to this compartment by toxic protein products, has been proposed to underlie the slow clearance of autophagosomes in the aging liver. Other studies in rodent liver have identified alterations in the response of macroautophagy to changes in circulating levels of insulin and glucagon with age. In young organisms, the increase in glucagon levels during starvation upregulates macroautophagy, whereas insulin, secreted as nutrients are absorbed in the gut, enhances the mTOR-mediated repression of this pathway. The inability of glucagon to upregulate macroautophagy in aging livers has been proposed to result from the basal signaling activity of the insulin receptor with age, which is also enhanced by oxidative stress in the absence of insulin. Macroautophagy deregulation in old organisms could also be a consequence of its persistent activation because of the gradual resistance to insulin, typical of aging cells. Although increased autophagy could be initially beneficial, its maintained activation could lead to chronic depletion of essential autophagic components and failure to upregulate this pathway when needed.

Studies in rodent livers also provided the first evidence that caloric restriction, the only intervention known to effectively decelerate aging, prevented the decline of macroautophagy activity with age. Limited dietary intake decreases the incidence of age-related disorders and increases life span in numerous experimental models from yeast and invertebrates to mammals including primates. The logical connection between caloric restriction and autophagy stems from the findings that that autophagy is a catabolic process upregulated during nutrient deprivation and that tissues from calorically restricted animals contain markedly lower levels of damaged components when compared to age matched ad libitum fed animals. Both roles of autophagy, as an energy source when nutrients are scarce and as a process to remove cellular damage, fit well with the beneficial effect of caloric restriction. The age-related decrease in the other nutritionally regulated autophagy pathway - CMA - was also described before its molecular components were identified. Early studies in cultured primary human fibroblasts revealed that the degradation of a selective pool of cytosolic proteins in lysosomes (through what is now known as CMA) was gradually impaired during passage in culture. This decrease in CMA in aging human fibroblasts was later confirmed in different tissues of old rodents.

The new connections between autophagy, aging and life span

Genetic screens in yeast (in the case of macroautophagy) and novel biochemical approaches (in the case of CMA) have helped to identify the subset of genes and protein products that participate as effectors and modulators of these autophagic pathways. Manipulations in these genes have confirmed the tight connection between autophagy, life span and aging. The first genetic connection between autophagy and aging was established in the worm Caenorhabditis elegans, an organism extensively used to analyze changes in protein degradation with age, in addition to the identification of genes that mediate life-span extension. Mutations in different genes that reduce signaling through the equivalent of the insulin-signaling pathway in worms, such as daf-2, can extend longevity. Knock-down of essential autophagy proteins in daf-2 mutants drastically reduced their life-span extension. Functional autophagy has also proven necessary to attain the maximal life-span extension mediated by deletion of other genes such as those encoding mTOR or the tumor suppressor p53, which curiously represses macroautophagy in mammals. Likewise, factors that promote longevity in invertebrates, such as the Foxo family of Forkhead transcription factors, upregulate macroautophagy. Indeed, macroautophagy activation is a common feature of all the long-lived mutant worms. In contrast to the well-established role of autophagy in the 'superlongevity' of these mutant worms, the requirement of functional autophagy for normal life span remains a controversial matter in this model. Thus, whereas mutation of essential autophagic genes shortened the life span of wild-type worms in some of these studies, it had no effect in others. The reasons for these discrepant results remain to be elucidated. Regardless of this controversy, accumulation of intracellular damage and signs of cellular aging have been observed in both worms and flies defective for essential autophagy genes.

The role of macroautophagy in life-span extension mediated by caloric restriction was recently genetically confirmed in C. elegans. Macroautophagy is upregulated in feeding-defective worm mutants used to model dietary restriction and disruption of essential autophagy genes shortened their life span. This is an interesting finding because caloric restriction further increases life span in the long-lived insulin-signaling mutants, indicating that both interventions influence aging by different mechanisms. Macroautophagy, thus, reveals itself as a possible common effector of both pathways, a role probably attributable to the dual regulation of macroautophagy by insulin-signaling and mTOR. Mutations in the TOR pathway increase life span in invertebrates, but these mutants no longer respond to caloric restriction, thereby supporting the idea that attenuation of TOR signaling is part of the downstream mechanism involved in the beneficial effects of caloric restriction. TOR is a negative regulator of macroautophagy that signals as a downstream kinase of the insulin-signaling pathway. However, insulin-independent signaling through TOR is possible because this kinase is also activated by nutrients and growth factors. Blockage of this last signaling mechanism is probably responsible for maintained activation of macroautophagy during caloric restriction. Interestingly, although macroautophagy is required for life-span extension in all these long-lived worm mutants, at least in worms, it is not sufficient and it probably acts in parallel with other downstream pathways.

The identification of the different molecular components of CMA has facilitated a better understanding of the reasons for the declined activity of this type of autophagy with age. Indeed, a step-by-step comparative analysis of CMA in livers of young and old rodents revealed that substrate recognition by the cytosolic chaperones, targeting to the lysosomal membrane and degradation in the lysosomal lumen are preserved until late in life. The problem, however, lies in the binding and lysosomal translocation of the substrate proteins because of progressively lower levels of the CMA receptor at the lysosomal membrane with age. This defect - also confirmed in other tissues of old rodents and in human aging fibroblasts - is not because of transcriptional down regulation of this receptor, but instead it is a consequence of instability of this protein once it reaches the lysosomal membrane. Changes with age, probably in the lipid composition of the lysosomal membrane, are behind the instability of the CMA receptor, LAMP-2A, in old organisms.

Consequences of autophagic failure in aging

In light of the myriad of physiological functions of autophagy, it is easy to infer that the described age-related decline in autophagic activity will affect normal cell functioning and contribute to different aspects of the aging phenotype. Recent studies using tissue-specific conditional autophagy-knockout mice have confirmed the important role of this catabolic process in the maintenance of cellular homeostasis and proper response to stress. Thus far, changes in life span and health span have not been analyzed in these rodents with impaired autophagy. However, worms and flies with defective autophagy have decreased life span and earlier features of cellular aging. An important limitation of the studies both in invertebrates and in mammals is that autophagic blockage is induced early in life, whereas the age-dependent decrease in this pathway does not begin in most organisms until middle age. It is, thus, possible that compensatory mechanisms that can be activated early in life in response to the autophagic failure are no longer possible late in life, resulting in even more dramatic consequences. In support of this possibility, recent studies in mouse models with temporally controlled knockout of an essential autophagy gene in the heart have revealed that autophagic impairment early in development does not result in altered heart function, unless these animals are exposed to additional stress. By contrast, autophagic blockage late in adulthood results in marked heart failure, even under normal resting conditions. Future studies on the consequences of macro autophagic decline with age should take into account these temporal differences.

The use of LAMP-2A knockdown in cultured fibroblasts to reproduce the age-related decline in CMA activity has confirmed the importance of this pathway in the removal of altered cytosolic proteins and as part of the cellular response to stress. However, selective blockage of CMA in a whole organism, to analyze the functional consequences of the accumulation of damaged products observed in the cultured cells, has not been possible yet. The main limitation in this respect is the fact that LAMP-2A is one of the three spliced variants of the lamp2 gene and, consequently, knockout of the whole gene would present a more complex phenotype than that resulting only from defective CMA. The use of RNAi against LAMP-2A in animals could help overcome this limitation in the future. Recently, particular attention has been dedicated to the consequences of reduced macroautophagy and CMA with age in the progression of the so called age-related disorders. The fact that alterations in cellular homeostasis or in the cellular response to stress are common to many of these late-onset disorders explains why alterations in autophagy are often found to underlie the basis of these pathologies and/or act as aggravating factors in the course of the disease.

Autophagy and age-related disorders

Age-related disorders are a broad group of diseases of higher prevalence in the elder population for which aging is considered a risk factor (e.g. cancer, neurodegenerative diseases, metabolic disorders such as diabetes, etc.). Here, I provide some examples of those disorders for which the aggravating effect of aging in the progression of the disease has been proposed to be, at least in part because of age-related changes in autophagy.

Neurodegenerative disorders

Alzheimer's disease (AD) and Parkinson's disease (PD), the two neurodegenerative disorders of highest prevalence in our society, are both late-onset diseases. Alterations in macroautophagy have been identified as one of the early changes in AD affected neurons, whereas a primary defect in CMA has been described in PD. Neurons respond to autophagic failure by up-regulating other proteolytic mechanisms (including autophagic pathways not affected by the disease). It has been proposed that this compensatory stage could be compromised with age because of the functional decline of the different autophagic pathways. This defect would eventually result in accumulation of pathogenic products, cellular death and on-set of symptoms related to loss of the affected neurons.

Cancer

Autophagy acts in tumor suppression by removing damaged organelles and reducing chromosome instability. As autophagic activity decreases with age, accumulation of intracellular damage, dysfunctional organelles and chromosome aberrations increase the chances of oncogenic transformation of somatic cells.

Immuno-senescence

Different aspects of the gradual deterioration of the immune system with age could be related to autophagic dysfunction. Decreased autophagy in professional antigen presenting cells (i.e. dendritic cells, macrophages and B-cells) could lead to inefficient presentation of exogenous antigens, poor T-cell activation and failure to orchestrate a proper immune response to pathogens. Furthermore, the inability of non-professional cells to present their own antigens through sampling of the cellular milieu by macroautophagy could underlie the abnormal autoimmune response sometimes observed in aging organisms.

Myopathies

Defective autophagosome clearance in the aging muscle could contribute to muscle wasting (sarcopenia) characteristic of old organisms. As autophagic vacuoles accumulate with age, they interfere with the contractile properties of muscle fibers weakening them and favoring small ruptures by traction, which eventually could lead to muscle atrophy.

Autophagy and fasting

Autophagy was first described in 1962 when researchers noted an increase in the number of lysosomes (the part of the cell that destroys stuff) in rat liver cells after infusing glucagon. The Nobel Prize winning scientist Christian de Duve coined the term autophagy. Damaged sub cellular parts and unused proteins become marked for destruction and then sent to the lysosomes to finish the job. One of the key regulators of autophagy is the kinase called mammalian target of rapamycin (mTOR). When mTOR is activated, it suppresses autophagy, and when dormant, it promotes it. The same process also happens at a sub-cellular level. You don't necessarily need to replace the entire car. Sometimes, you just need to replace the battery, throw out the old one and get a new one. This also happens in the cells. Instead of killing off the entire cell (apoptosis), you only want to replace some cell parts. That is the process of autophagy, where sub-cellular organelles are destroyed and new ones are rebuilt to replace it. Old cell membranes, organelles and other cellular debris can be removed. This is done by sending it to the lysosome which is a specialized organelle containing enzymes to degrade proteins.

Nutrient deprivation is the key activator of autophagy. Remember that glucagon is kind of the opposite hormone to insulin. It's like the game we played as kids - 'opposite day'. If insulin goes up, glucagon goes down. If insulin goes down, glucagon goes up. As we eat, insulin goes up and glucagon goes down. When we don't eat (fast) insulin goes down and glucagon goes up. This increase in this is in essence a form of cellular cleansing. The body identifies old and substandard cellular equipment and marks it for destruction. It is the accumulation of all this junk that may be responsible for many of the effects of aging. glucagon stimulates the process of autophagy. In fact, fasting (raises glucagon) provides the greatest known boost to autophagy. Fasting is actually far more beneficial than just stimulating autophagy. It does two good things. By stimulating autophagy, we are clearing out all our old, junky proteins and cellular parts. At the same time, fasting also stimulates

growth hormone, which tells our body to start producing some new snazzy parts for the body. We are really giving our bodies the complete renovation.

You need to get rid of the old stuff before you can put in new stuff. Think about renovating your kitchen. If you have old, crappy 1970s style lime green cabinets sitting around, you need to junk them before putting in some new ones. So the process of destruction (removal) is just as important as the process of creation. If you simply tried to put in new cabinets without taking out the old ones, it would be pretty fugly. So fasting may in some ways reverse the aging process, by getting rid of old cellular junk and replacing it with new parts. Autophagy is a highly regulated process. If it runs amok, out of control, this would be detrimental, so it must be carefully controlled. In mammalian cells, total depletion of amino acids is a strong signal for autophagy, but the role of individual amino acids is more variable. However, the plasma amino acid levels vary only a little. Amino acid signals and growth factor / insulin signals are thought to converge on the mTOR pathway - sometimes called the master regulator of nutrient signalling. So, during autophagy, old junky cell components are broken down into the component amino acids (the building block of proteins). What happens to these amino acids? In the early stages of starvation, amino acid levels start to increase. It is thought that these amino acids derived from autophagy are delivered to the liver for gluconeogenesis. They can also be broken down into glucose through the tricarboxylic acid (TCA) cycle. The third potential fate of amino acids is to be incorporated into new proteins.

The consequences of accumulating old junky proteins all over the place can be seen in two main conditions - Alzheimer's disease (AD) and cancer. Alzheimer's disease involves the accumulation of abnormal protein - either amyloid beta or Tau protein which gums up the brain system. It would make sense that a process like autophagy that has the ability to clear out old pro What turns off autophagy? Eating. Glucose, insulin (or decreased glucagon) and proteins all turn off this self-cleaning process. And it doesn't take much. Even a small amount of amino acid (leucine) could stop autophagy cold. So this process of autophagy is unique to fasting - something not found in simple caloric restriction or dieting.tein could prevent the development of AD. There is a balance here, of course. You get sick from too much autophagy as well as too little. Which gets us back to the natural cycle of life - feast and fast. Not constant dieting. This allows for cell growth during eating, and cellular cleansing during fasting - balance. Life is all about balance [23].

In the endless debates over what-to-eat and what-not-to-eat, consideration of when to eat too often gets overlooked. This is unfortunate - research shows pretty convincingly that timing our meals intelligently can produce remarkable health benefits. I'm referring specifically to fasting, defined loosely as the practice of abstaining from food for periods ranging anywhere from 12 hours to several weeks. Intermittent fasting (IF) - the practice of regularly reducing calorie intake to zero for periods of 12-24 hours - continues to gain popularity with many IF-practitioners experiencing dramatic (and largely effortless) improvements in cognitive function and/or body composition[24]. Muslims always fasting one month in a year for more than 12 hours with Zero intake of food so cancers and Alzheimer's disease are not common among them. For the past 4 years or so I've been following the most popular version of IF; restricting (almost) all of my calorie consumption to a 6-hour window each day (thus doing a daily 18-hour "fast"). The benefits of this practice are profound, and it's probably the single most impactful dietary practice I've adopted in the past 5 years. Recently, however, after diving into newer research elucidating the mechanisms behind the benefits of fasting, I've changed the timing and structure of my fasts a bit in an effort to maximize the activity of these mechanisms. The 2016 Nobel Prize in Physiology or Medicine went to a Japanese gentleman by the name of Yoshinori Ohsumi for his discoveries of the mechanisms behind autophagy, a cellular maintenance process - stimulated by fasting - that is critical in disease resistance, longevity and general body and brain vitality.

To vastly (but not inaccurately) oversimplify: healthy cells are actively autophagic, unhealthy cells are not. The changes I've made to my own fasting protocol were specifically intended to increase stimulation of autophagy. Before going into these let's first look at the ridiculously-long list of beneficial metabolic and hormonal processes stimulated by fasting, which should make it pretty clear why I consider fasting to be an indispensable practice for anyone that values body and brain performance. Improved Brain Health/Cognition - There are a set of metabolic processes neurologists will tell you are essential for maintaining a healthy, high-performing brain, and fasting stimulates essentially all of them. Fasting increases circulating levels of several neurotrophic factors, biomolecules that support the growth, survival, and differentiation of neurons. The result is enhanced network plasticity (critical for learning), increased stress resilience and increased mitochondria (i.e. increased cognitive energy). Fasting also reduces oxidative stress (and thus, inflammation) in the brain both by stimulating the removal of damaged molecules and stimulating production of endogenous antioxidants. All of these translate to meaningful improvements in brain performance. Fasting

has also been shown to reduce the neuronal dysfunction that results from Alzheimer's, Parkinson's and other neurodegenerative diseases. Slowing/Reversing Markers Of Aging - A common way biochemists define aging is as "the slow accumulation of dysfunctional proteins and organelles in our cells" - which leads eventually to cell dysfunction and/or death. Owing largely to the stimulation of autophagy, fasting can reverse this process, stimulating cells to "clean house", preventing the dysfunction that can lead to disease (including cancer).

Improved Body Composition - There's a lot of confusion out there around the factors that determine an individual's body. Most people - including many nutritionists - incorrectly assume changes in body composition are largely attributable to the calories-in-calories-out model of diet and exercise. In reality, body composition is largely a function of our hormonal state. Fasting increases insulin sensitivity and increases adiponectin levels, two key hormonal factors that determine if existing fat gets oxidized (used for energy) as well as if incoming caloric energy gets used immediately or stored (as fat) for future use. These positive hormonal changes persist well after a fast is completed. So while you will likely run a calorie deficit on fasting days, the hormonal changes will have a far bigger impact on body composition over weeks and months. A lot of gym-goer-types assume that fasting will also lead to the body breaking down muscle for energy, and while this could be true under certain conditions, it's also fairly easy to avoid with a bit of strategy, which I'll outline later. Done smartly, short term fasting increases lipolysis (fat burning) while largely maintaining muscle.

Improved Digestion - Intuitively, fasting acts as a sort of "digestive reset" allowing the gastrointestinal (GI) tract relax for a bit. In practice, this produces both reduced intestinal inflammation and improved motility (the contraction of GI muscles in digestion). Both lead to improved nutrient absorption and better bowel movement quality. Interestingly, a recent study showed that fasting might stimulate the growth of specific species of bacteria in the gut that promote lipolysis (fat burning). Cardiovascular Health - Fasting reduces resting heart rate and blood pressure while increasing parasympathetic tone (an important indicator for health of the cardiovascular system). In general, the resilience of the cardiovascular system to stress is improved by fasting. Cancer Prevention/Treatment - Talk to most anyone involved in research around calorie restriction or fasting and they'll tell you these are tragically underused tools in the cancer treatment toolkit. Fasting has been shown to comparable in efficacy to chemotherapy in delaying the growth of certain types of tumors. Think about that: fasting is comparable in efficacy to the unbelievably toxic chemical soup that works by (hopefully) killing cancerous cells ever-so-slightly faster than it kills the recipient. Why is this not more used in oncology? At minimum, a fasting protocol should be used in addition to chemotherapy, as it has been shown to preferentially protect non-cancerous cells from chemo drugs.

Autophagy deserves special attention here, as a case can be made it's the single most important metabolic process to select for if the aim is to slow the aging process and promote a high-performing body and brain. You don't win a Nobel Prize in medicine unless you're working on something that legitimately has the potential to change humanity. Autophagy is still relatively obscure outside the biochemistry/cell bio/endocrinology worlds, but my intuition (and hope) is that it will receive increasing attention in mainstream natural health and nutrition media over the next 5 years or so. As Yoshinori Ohsumi and others have described, autophagy is the process by which cells degrade and then recycle unneeded or dysfunctional proteins and organelles (via lysosomes). If allowed to accumulate, dysfunctional proteins and organelles eventually lead to dysfunctional cells that either die, persist as dysfunctional cells (contributing to poor tissue/organ function) or become cancerous. Needless to say, all of these outcomes are in opposition to a youthful, vibrant, high-performing body and brain. In the brain, upregulation of autophagy is strongly neuroprotective while disruption of autophagy causes neurodegeneration. In the liver, upregulation of autophagy increases lipolysis (fat usage) and insulin sensitivity, while disruption of autophagy leads to prediabetes and metabolic syndrome. The list goes on, but I think you get the point: autophagy is important.

Fasting to maximize autophagy

Fasting has been shown to be, far and away, the most effective way to stimulate autophagy in both the body and brain. Despite this, there's not a clear consensus on exactly how long to fast for to maximize autophagy. I located a few studies that looked at the level of autophagy occurring in both the liver and brain, and there were some clear patterns when looking at the data of each. The level of autophagy activity can be measured by simply counting the number of autophagosomes (the cellular organelles that degrade dysfunctional proteins), as these will increase in number when autophagy is stimulated. The study looking at liver cells found that the number of autophagosomes increased 300% after 24 hours of fasting, and a further 30% after 48 hours of fasting. Studies looking at autophagosomes in brain cells had a similar findings. In addition to looking at the number of autophagosomes in a cell, the brain study looked at a handful of metabolic markers that are indicative of autophagy being stimulated. Almost of these markers peaked between 24 and 36 hours, explaining why the increase in autophagosomes between 24 and 48 hours is substantially less than between hours 0 and 24. My take-away from this is that while there is certainly value in fasting longer, there also seems to be an element of diminishing returns once a fast passes the 36 hour mark. As such, I've designed my fasting protocol to get close to the 36 hour mark with as little stress and discomfort as possible. More on this in a bit.

Psychological benefits

A quick word on the utility and value of fasting. It feels incomplete to express the benefits of fasting simply as

"stimulation of autophagy" or "increased neurotrophic factors", etc. Talk to anyone who fasts on a regular basis and it will be clear that there is more to derive from the experience than simply an abstract understanding that you're "doing something healthy." There is an undeniable physical and emotional high that comes with fasting. Almost everyone gets this the day following a fast, and for a lot of people, there is a high in the fasting period that outweighs the slight discomfort of an empty stomach. I think the reason for this it two-fold - yes, stimulating dozens of vitality-promoting metabolic processes surely contributes to the sense of wellbeing, but I think the psychological component is even more important. Fasting requires an element of self-mastery. Most of us are conditioned both psychologically and hormonally to be eating multiple times during the day, and any deviation from this causes people to get cranky. This is not healthy. Fasting also requires us to be present with the initial discomfort of not stuffing our faces every time we feel like it. This is a reversal of the typical power structure in body-brain relations. It puts us in the position of consciously making the decision to eat or not to eat, rather than our stomach (aka hormones) effectively making that decision for us. Almost all of us would benefit hugely from consciously redefining our relationship to food, and fasting is a powerful opportunity to do this redefinition.

Autophagy and Exercise

Exercise induces autophagy in peripheral tissues and in the brain

We recently identified physical exercise as a newly defined inducer of autophagy *in vivo*. Exercise induced autophagy in multiple organs involved in metabolic regulation, such as muscle, liver, pancreas and adipose tissue. To study the physiological role of exercise-induced autophagy, we generated mice with a knock-in nonphosphorylatable mutation in BCL2 (Thr69Ala, Ser70Ala and Ser84Ala) (BCL2 AAA) that are defective in exercise- and starvation-induced autophagy but not in basal autophagy. We found that BCL2 AAA mice could not run on a treadmill as long as wild-type mice, and did not undergo exercise-mediated increases in skeletal glucose muscle uptake. Unlike wild-type mice, the BCL2 AAA mice failed to reverse high-fat diet-induced glucose intolerance after 8 weeks of exercise training, possibly due to defects in signaling pathways that regulate muscle glucose uptake and metabolism during exercise. Together, these findings suggested a hitherto unknown important role of autophagy in mediating exercise-induced metabolic benefits. In the present addendum, we show that treadmill exercise also induces autophagy in the cerebral cortex of adult mice. This observation raises the intriguing question of whether autophagy may in part mediate the beneficial effects of exercise in neurodegeneration, adult neurogenesis and improved cognitive function.

The relationship among autophagy, exercise and metabolic regulation has been a largely unexplored field. Physical exercise has numerous health benefits, such as life-span expansion, and protection against cardiovascular diseases, diabetes, cancer and neurodegenerative diseases. Many of these health benefits overlap with known protective functions of the cellular pathway of macroautophagy (herein referred to as autophagy). Thus, we proposed that some of the health benefits of exercise may be due to autophagy activation. To test this hypothesis, we exercised wild-type mice that transgenically express the fluorescent autophagy marker GFP-LC3 on a treadmill, using a running protocol with increasing speed at defined intervals. We found that in both skeletal and cardiac muscle 30 min of exercise was sufficient to induce GFP-LC3 puncta (autophagosome) formation, which reached a plateau after 80 min. Using a combination of assays including GFP-LC3 puncta formation, LC3-II conversion and SQSTM1/p62 degradation, we showed that autophagy activity is induced by exercise in multiple organs, including skeletal muscle, heart, liver, pancreatic cells and adipose tissue. These observations suggested a possible role of autophagy in metabolic regulation during exercise. To study specific roles of exercise-induced autophagy, we utilized a mouse model that is defective in exercise-induced autophagy but maintains normal levels of basal autophagy. Previous reports have shown that loss of basal autophagy activity in cardiac or skeletal muscle leads to abnormal development, and cardiac failure and skeletal muscle atrophy, respectively; therefore, (inducible) tissue-specific knockout of autophagy genes in these organs would not be a suitable approach for examining the physiological effects of deficient exercise-induced autophagy. Thus, we generated a new mouse model (BCL2 AAA mice) with a knock-in mutation in the phosphorylation sites of the nonstructured loop of the anti-autophagy protein, BCL2. In a previous study, we found that multisite phosphorylation of BCL2 is essential for its release from BECN1 (also known as Beclin 1) and for starvation-induced autophagy *in vitro*, and that nonphosphorylatable mutations in human BCL2 block starvation-induced, but not basal, autophagy. As expected, we found that BCL2 AAA mice (which contain nonphosphorylatable mutations in the analogous sites in mouse BCL2) had normal muscle histology and normal levels of basal autophagy, but were defective in starvation- and exercise-induced autophagy. Accordingly, the BCL2 AAA mice serve as a useful model system to further study the functions of exercise-induced autophagy.

During acute exercise (single bout of treadmill running), compared with wild-type mice, BCL2 AAA mice had decreased exercise endurance and impaired increases in muscle glucose metabolism, including lower levels of decline in serum glucose and insulin levels, decreased plasma membrane relocalization of the SLC2A4 (also known as GLUT4) glucose transporter, decreased uptake of radiolabeled glucose, and decreased activation of AMP-activated protein kinase (AMPK). Similar findings were also observed in mice with mono-allelic loss of Becn1, and mice with hypomorphic expression of Atg16l1, suggesting that this phenotype was due to impaired autophagy activation, rather than an off-target effect of the BCL2 AAA mutation. These abnormalities in glucose metabolism during acute

exercise in mice deficient in exercise-induced autophagy led us to investigate whether autophagy might contribute to some of the beneficial metabolic effects of chronic exercise training. Exercise training protects against high-fat diet-induced type 2 diabetes in rodents and humans. We found that the induction of autophagy may be required for this protection, as only wild-type, but not BCL2 AAA, mice, reversed their dietary-induced glucose intolerance after 8 weeks of exercise training. We postulate that this mechanism may involve the impaired exercise-induced increase in muscle glucose uptake and AMPK activation that we observed during single bouts of forced treadmill exercise. Together, our findings suggested an unexpected role of autophagy in the regulation of glucose metabolism, AMPK activation, and exercise-mediated protection against type 2 diabetes. Given the central role of AMPK activation in treatment of diabetes and prevention of cancer, this positive feedback loop (also reported by others *in vitro*) between autophagy and AMPK activation during exercise may have important implications for understanding the role of altered autophagy in metabolic diseases, cancer and aging.

Intriguingly, in addition to various peripheral organs involved in metabolism, such as muscle, liver, pancreas and adipose tissue, we found that autophagy is also potently induced by acute exercise in the brain. We exercised 8-week-old GFP-LC3 transgenic wild-type mice on the treadmill for 95 min, and examined biochemical markers of autophagy by western blot analyses, as well as GFP-LC3 puncta in brain sections after paraformaldehyde perfusion by fluorescence microscopy. We found that there was a 2-fold increase in numbers of GFP-LC3 puncta in the anterior cerebral cortex after exercise. Importantly, we note that there is a very high level of auto-fluorescence in brain sections as compared with other tissues, which is perhaps why this fluorescent autophagy reporter has not been used extensively to study autophagy in the brain in prior studies (despite widespread use in other tissues). However, we found that the use of spectral unmixing (which separates the wavelengths of background auto-fluorescence from the wavelength of GFP) allowed us to detect authentic GFP-LC3 puncta. We also confirmed this finding by performing immunostaining with an anti-GFP antibody. Of note, we did not detect significant increases in GFP-LC3 puncta in regions of the brain besides the cerebral cortex, including the olfactory bulb, hypothalamus, midbrain or cerebellum (data not shown). We cannot rule out the possibility that other methods of sample preparation, similar to those used in recent reports that have detected increased GFP-LC3 puncta in the brains of mice subjected to starvation, might be more sensitive and detect autophagy induction in other regions of the brain. In addition to increased GFP-LC3 puncta, we detected a marked decrease in SQSTM1/p62 levels in whole brain lysates and cerebral cortex lysates from wild-type mouse brains after exercise, which was not observed in the brains of BCL2 AAA mice after an identical duration and intensity of treadmill exercise. These data suggest that autophagic flux in brain is increased after exercise in wild-type mice but not in BCL2 AAA mice. We did not detect changes in LC3-II conversion pre- and post- exercise in either genotype in whole brain, or in cerebral cortex. This may reflect decreased sensitivity of the LC3-II conversion assay as compared with measurements of SQSTM1/p62 degradation or GFP-LC3 puncta. It is also possible that there are other Atg8 homologs in the brain that may play more important roles than LC3B in autophagy, and that the anti-LC3B antibody used for western blot analysis in our study therefore did not detect conversion of the biologically relevant Atg8 homolog. Further studies will be required to elucidate in more detail the role of lipidation of specific mammalian Atg8 homologs in exercise-induced autophagy in the brain. Taken together, we conclude that exercise induces autophagy in the cerebral cortex of the brain. More detailed analyses will be required to determine the precise subpopulations of neurons that upregulate autophagy in response to exercise. Previous studies have shown that treadmill exercise upregulates sirtuin 1 levels and AMPK activation in rat brain. As both of these factors function as positive regulators of autophagy, one obvious question is whether they may contribute to the upregulation of autophagy that we observed in the brains of wild-type mice after exercise. There are numerous additional questions that remain to be answered. For example, the precise molecular mechanisms that cause altered glucose metabolism in autophagy-deficient mice remain unknown. Another open question is whether exercise-induced autophagy contributes to the beneficial effects of exercise on diseases other than diabetes, such as aging, cancer, cardiovascular diseases, inflammatory diseases and neurodegenerative diseases. Based on our new data that exercise can induce autophagy in certain regions of the brain, it will be important to investigate the physiological consequences of this phenomenon. Of note, autophagy is an important **"**housekeeping**"** mechanism that eliminates protein aggregates and damaged organelles in neurons, and exercise is an intervention that improves neuronal synaptic plasticity, promotes adult neurogenesis, prevents cognitive decline in aging, and delays the onset of neurodegenerative diseases. Thus, a crucial question is whether exercise-induced autophagy in the brain mediates some of these neuroprotective effects. Another intriguing question is whether the signals that trigger exercise-induced autophagy in the brain are cell-autonomous or derived from extrinsic systemic cues.

Want to clean up cellular garbage? Train fasted!

Exercise is a stressor. It modifies the intra and extracellular milieu, impairs the energetic status and stretches, sometimes even over-stretches the membranes. That certainly sounds as if you want to avoid it at all costs, but as nature had it, it is this eustress (good stress) that is absolutely essential for the remodeling of the muscle we are all working out for to happen - no stress no reason to adapt. It's that simple and does still have one major caveat: Too much stress and the adaptation turns into a constant and often insufficient repair process.

But who wants to "deconstruct" muscle, anyway?

Now, from the gymbro perspective the most important aspect of the training induced adaptation processes would

probably be protein synthesis. And while you can actually argue that this was the case if things were just about "growing", a different picture emerges, when you look at health benefits and the actual remodeling process which does necessarily begin with "demodeling", or rather the demolution of old muscle tissue - when that's happening in a controlled self-induced (by the cell) manner, scientists call this process autophagy.

Autophagy is one of the main reasons fasting is good for you

Now, when cells "kill" themselves, they usually do that for a reason. In fact, the process of autophagy must be seen as part of the general housekeeping - a part with enormous importance, as one of the possible consequences of its failure is cancer. Moreover, it has been demonstrated only recently that autophagy is also an essential process for muscle adaptation: "Autophagy is activated in skeletal muscle by numerous catabolic stimuli such as food deprivation, denervation or sepsis. However, evidence for the necessity of basal autophagy level in the maintenance of myofibrillar integrity has counterbalanced the vision of a system only implicated in muscle wasting. Very recently, the activation of the autophagy-lysosomal pathway has emerged as an essential process for skeletal muscle adaptation after endurance training.

In 2011 van Proeyen et al. [observed that training fasted does not only increase the intramuscular fatty acid oxidation in 20 healthy young volunteers, it did also and this may come as a surprise, prevent "the development exercise-induced drop in blood glucose concentration, the same drop in blood sugar that will make you feel exhausted and is a potential risk factor for an acceleration of the metabolic down regulation that occurs, whenever you are dieting. One year before van Proeyen et al. had already established that in times of high fat overfeeding (+30%kcal; 50% fat) only fasted training was able to increase the AMPK levels (=anti-cancer, anti-diabetic, anti-obesity effect) in young men. Said study by van Proyen was by the way the first to prove that fasted training is more potent than fed training to facilitate adaptations in muscle and to improve whole-body glucose tolerance and insulin sensitivity during hyper-caloric fat-rich diet. So, I suggest you remember it, when you wake up tomorrow and think about whether you should go for a run now or rather after filling up your belly with some delicious eggs or whatever it is that you have for breakfast.

References

1. Noboru Mizushima (2007) Autophagy: The Powerful of Immune Response. Genes & Dev 21: 2861-2873.
2. Lauren A Maggio (2013) Autophagy: The Powerful of Immune Response. J Med Lib 101(3): 205-212.
3. Makar AB, McMartin KE, Palese M, Tephly TR (1975) Autophagy: The Powerful of Immune Response. Biochem Med 13(2): 117-126.
4. Maloyan A, Muralimanoharan S, GaoX, Myatt L (2016) Autophagy: The Powerful of Immune Response. Autophagy 12(5): 752-769.
5. Liu N, Zhuang S, Xu L, Shi Y (2017) Autophagy: The Powerful of Immune Response. J Diabetes Res 3560238.
6. Hirotaka Watadahttps, Yoshio Fujitani (2015) Autophagy: The Powerful of Immune Response. Mol En 29(3): 338-348.
7. Palese M, Makar AB, McMartin KE, Tephly TR (1975) Autophagy: The Powerful of Immune Response. Biochem Med 13(2): 117-126.
8. Zeisel SH, da Costa KA (2009) Autophagy: The Powerful of Immune Response. Nutr Rev 67(11): 615-623.
9. http://www.fasebj.org/content/25/1_Supplement/1095.2
10. Satoru Kobayashi, Qiangrong Liang (2015) Autophagy: The Powerful of Immune Response. BBA 1852(2): 252-261.
11. Zhonglin Xie (2012) Cardiac Autophagy in Diabetic Cardiomyopathy. Bioenergetics 1: e110.
12. Sebastian Dabrowski, Christian Staat, Denise Zwanziger, Reine-Solange Sauer, Christian Bellmann, et al. (2014) Autophagy: The Powerful of Immune Response. ARS Central 22(1): 1-14.
13. Wittich CM, Burkle CM, Lanier WL (2012) Autophagy: The Powerful of Immune Response. Mayo Clin Proc 87(10): 982-990.
14. Makar AB, McMartin KE, Palese M, Tephly TR (1975) Autophagy: The Powerful of Immune Response. Biochem Med 13(2): 117-126.
15. Enomoto Mhttps, Tsuchida A, Miyazawa K, Kawakita H, Yokoyama T, et al. (2007) Autophagy: The Powerful of Immune Response. Int J Mol Med 20(6): 801-808.
16. Shaun Steele, Jason Brunton, Thomas Kawula (2015) Autophagy: The Powerful of Immune Response. Front Cell Infect Microbiol 5: 51.
17. Robin Mathew, Eileen White, Vassiliki Karantza-Wadsworth (2010) Autophagy: The Powerful of Immune Response. Nat Rev Cancer 7(12): 961-967.
18. Meng Li, Ping Gao, Junping Zhang, Robin, Vassiliki Karantza-Wadsworth (2010) Autophagy: The Powerful of Immune Response. Int J Mol Sci 17(3): 332.
19. Cuervo AM (2008) Autophagy: The Powerful of Immune Response. Trends Genet 24(12): 604-612.
20. https://www.dietdoctor.com/renew-body-fasting-autophagy
21. Graham Ryan (2017) The Profound Benefits of Fasting and Autophagy.
22. He C, Sumpter R, Levine B (2012) Autophagy: The Powerful of Immune Response. Autophagy 8(10): 1548-1551.
23. (2013) Want to Clean Up Cellular Garbage? Train Fasted! Fasted Training Boosts Cellular Housekepping (Autophagy) & Forms the Basis of Structural Adaptations to ERD.