Autophagy:
Historical, Molecular and Health Aspects

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CONTENTS

1. Introduction

Autophagy is an evolutionary highly conserved process in eukaryotes by which cytoplasmic materials reach lysosomes for degradation. These materials include macromolecules (like proteins or lipids) and organelles (like mitochondria or the endoplasmic reticulum ER) (Yang and Klionsky, 2010a; Mizushima and Levine, 2010).
The term “autophagy” is derived from the Greek words “auto” meaning self, and “phagy” meaning eating. The word “autophagy” has been coined by Christian de Duve, who won the 1974 Nobel Prize for his research on lysosomes (Ravikumar et al., 2010).
The first hypothesis why this kind of “self-eating” would occur was that autophagy disposes intracellular rubbish like misfolded proteins, but we have since learnt that autophagy is dynamically regulated, has various vital roles, and is induced by several stresses, including nutrient starvation, hypoxia, pharmacological agents and diseases.

So far, three forms of autophagy have been discovered: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). CMA involves cytosolic proteins, which are targeted and unfolded by chaperones, and selectively translocated across the lysosomal membrane. Only proteins are degraded by CMA. Microautophagy describes the direct engulfment of cytosolic content by invagination of the lysosomal membrane. Some research on microautophagy in yeast has been conducted, but it’s not well-characterised in mammals (Yang and Klionsky, 2010a; Mizushima, Yoshimori and Levine, 2010). This thesis will solely focus on macroautophagy (referred to as autophagy hereafter), a lysosomal delivery pathway which mediates the formation of double-membrane autophagosomes that fuse with lysosomes, forming the autolysosome in which the enwrapped cellular components are degraded (Figure 1) (Rubinsztein, Marino and Kroemer, 2011).

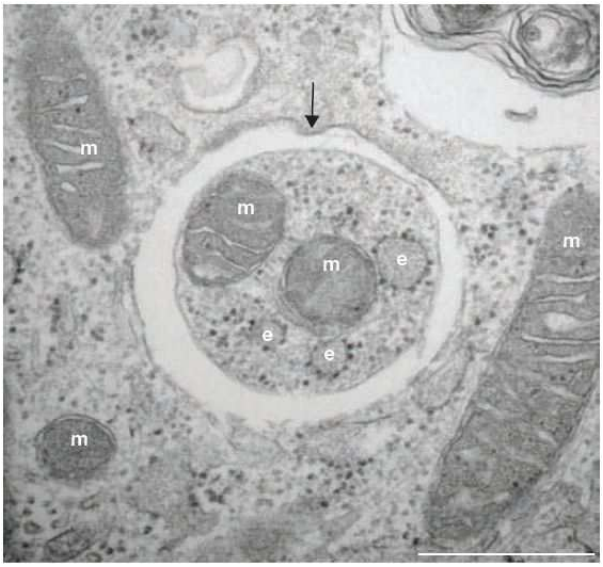

Figure 1. Mitochondria (m) and endoplasmatic reticulum (e) inside an autophagosome (arrow)
Electron microscopic analysis of mouse embryonic fibroblast during nutritient starvation. Scale bar, 10 µm. (Mizushima and Levine, 2010)

2. History

Clark (1957) and Novikoff (1959) observed so called “dense bodies” while examining mouse kidneys by electron microscope. These dense bodies were compartments containing mitochondria and lysosomal enzymes. In 1962, Ashford and Porter exposed rats to glucagon and reported semi-digested mitochondria and ER in membrane-bound vesicles of hepatocytes.

Findings by Novikoff and Essner showed that these vehicles contained lysosomal hydrolases. In 1963, de Duve used the word “autophagy” to describe vesicles containing disintegrated parts of the cytoplasm (as reviewed by Yang and Klionsky, 2010a). Four years later, Deter and de Duve, studying the appearance of autophagic vacuoles in rat liver after glucagon injection, found that the number of dense bodies was significantly reduced. More than 50% of dense bodies disappeared, the first evidence that autophagy is induced by glucagon (Deter, Baudhin and de Duve, 1967). In 1977, Pfeifer observed that autophagy is inhibited by insulin (as reviewed by Yang and Klionsky, 2010a).

Fifteen years later, Seglen and Gordon observed that autophagy is regulated by protein kinases and phosphatases. Seglen’s team examined the effect of okadaic acid (OA), a protein phosphatase inhibitor and various protein kinase inhibitors on hepatocytic autophagy. In the hepatocytes of starved rats, OA caused a dose-dependent inhibition of autophagy. KT-5962, a general protein kinase inhibitor, and KN-62, a specific CaMK-II inhibitior, antagonized and reversed the effect of OA. (Holen, Gordon and Seglen, 1992).
In 1994, Schreiber’s group identified a mammalian protein targeted by rapamycin kinase which they initially called FRAP (FKBP-rapamycin-associated-protein) (Brown et al., 1994). FRAP, today usually called TOR (target of rapamycin kinase) was the key to understand the signalling pathways regulating autophagy.

The identification of the first autophagy-related gene (ATG) in yeast by Ohsumi’s group in 1997 was a major breakthrough. The group reported a structural analyses of the APG1 gene and it’s gene product, Apg1p. Apg1p encodes a Ser/Thr-type protein kinase, whose autophosphorylation activity drops during starvation (Matsuura, Tsukada, Wada and Ohsumi, 1997). One year later, the first report of mammalian autophagy genes (ATG5 and ATG12) came from the same research team. Their observations indicated that the Atg12-Atg5 conjugation system is conserved (Mizushima, Sugita, Yoshimori and Ohsumi, 1998).

The identification of protein 1 light chain 3 (LC3, also known as MAP1LC3), the mammalian homologue of yeast Atg8 by Kabeya, while in Yoshimori’s laboratory in the year 2000, was highly important for further research, since it enabled the development of LC3-based assays to examine autophagy in higher eukaryotes. A study on LC3 provided Yoshimori and his colleagues with the opportunity to demonstrate that LC3 is critical for autophagosome formation, associates to the autophagosomal membranes (the first and so far only protein known to do so) and exists in two different forms in cells. They observed that LC3-I is located in the cytosolic fraction from liver homogenate of starved rat, while LC3-II is starvation-dependent and enriched in the autophagic vacuole fraction. Kabeya proposed LC3-II as “a good marker for autophagosomes” (Kabeya et al., 2000).

Recently, the complexity of the human autophagy system has been demonstrated by Behrends et al., who conducted a large-scale screen in human cells. While for example the atg8p in yeast is represented by a single gene, there are six human ATG8 orthologues (LC3/GABARAP proteins), which interact with 67 other proteins. Using retrovirally expressed Flag-haemagglutinin fusion proteins, LC-MS/MS and proteomics analysis software, the research group detected and calculated a total of 409 HCIPs (high confidence interaction proteins) making 751 interactions (Behrends, Sowa, Gygi and Harper, 2010).

3. Molecular machinery

3.1 Overview

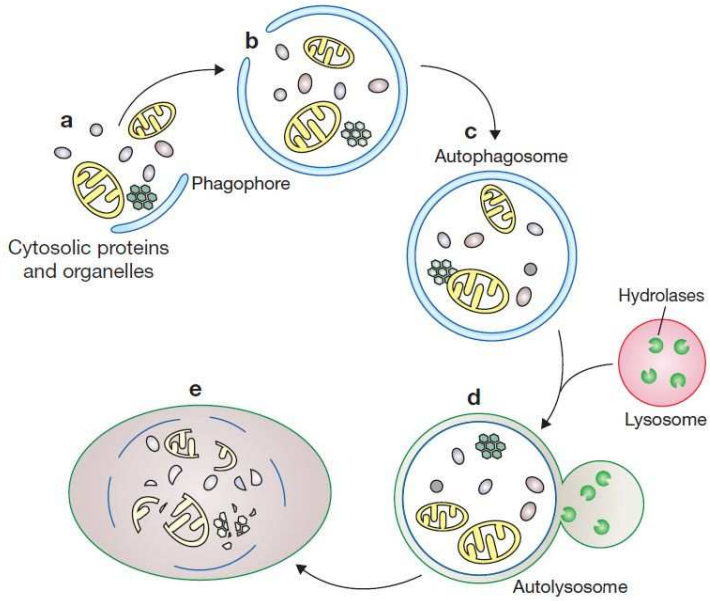

Figure 2. Schematic depiction of the autophagy pathway
(Xie and Klionsky, 2007)

As depicted in figure 2, mammalian autophagy proceeds through a series of steps, and is believed to start at the phagophore assembly site (PAS) (a). After formation of the phagophore (sometimes called the isolation membrane) at the PAS, the double membrane expands, enwraps cellular components (b) and closes to form a vesicle, the autophagosome (c). Fusion of an autophagosome with a lysosome containing hydrolases creates the autolysosome (d), where the inner membrane and cargo are broken down and recycled (e) (Yang and Klionsky, 2010b).

3.2 Core molecular machinery

In yeast, more than 30 autophagy-related genes (ATG) have been reported, and several homologs in higher eukaryotes were identified. ATG gene products are essential for macro- and microautophagy. Among these ATG genes, 15 are referred to as “core” genes since their Atg proteins are required for autophagosome formation. (Bhatia-Kiŝŝová and Camougrand, 2010).

The Atg proteins forming the core machinery are composed of four different functional groups (Yang and Klionsky, 2010b):

- Atg9/mAtg9 transmembrane proteins and its cycling system of associated proteins
- Atg12 and Atg8/LC3 ubiquitin-like protein conjugation system
- Atg6/Atg14 class III phosphatidylinositol 3-OH kinase (PtdIns3K) complex
- Atg1/unc-51-like kinase (ULK) complex

These proteins are recruited to the phagophore assembly site (PAS). The PAS is made up of the phagophore and the core machinery proteins, which expand and transform the phagophore into an autophagosome (Figure 3). All proteins except Atg8/LC3 are excluded from the completed vesicle (Xie and Klionsky, 2007).

Atg9 is a transmembrane protein that cycles between the trans-Golgi network and endosomes. The movement of Atg9 between the PAS and other cytosolic components is necessary for authophagosome formation, Atg9 is probably delivering membrane for expansion of the autophagophore (Ravikumar et al., 2010). VMP1 (vacuole membrane protein 1) is the second transmembrane protein essential for mammalian autophagy that has been discovered.
Human Atg9 homologues were observed at the trans-Golgi network and endosomes, in contrast to yeast, where non-PAS Atg9 was found at the surface of mitochondria. The delivery of Atg9 to the PAS depends on the two transport factors Atg23 and Atg27, retrieval of Atg9 requires Atg2, Atg18 and Atg1. Atg23 is a soluble protein, Atg2, Atg18 and Atg27 are membrane proteins, Atg1 is a serine-threonine protein kinase which regulates Atg9 trafficking from the PAS (Xie and Klionsky, 2007).

Atg8/LC3 and Atg12 are ubiquitin-like proteins. Despite no obvious similarity to ubiquitin in their primary sequence, crystal structures of their mammalian homologues show that both have a ubiquitin fold at the C terminus (Xie and Klionsky, 2007). The C terminus of Atg8/LC3 is cleaved by the cysteine protease Atg4, exposing the glycine residue and generating the cytosolic LC3-I. The E1-like activating enzyme Atg7 and the E2-like conjugating enzyme Atg3 attach LC3-I to phosphatidylethanolamine (PE), forming the lipidated LC3-II. LC3-II integrates into both the internal and external side of the phagophore membrane. This specific association makes it an excellent marker for studying autophagy (Mizushima, Yoshimori and Levine, 2010).
In a similar reaction requiring Atg7 (E1-like activating enzyme) and Atg10 (E2-like conjugating enzyme), the C-terminal of Atg12 is attached to a lysine in Atg5. Atg12-Atg5 oligomerizes with Atg16 to the multimeric Atg16L complex. Atg16L is localized at the outer phagophore membrane where it acts as a E3-like ligating enzyme, which is essential for the elongation of the autophagosomal membrane (Yang and Klionsky, 2010b).

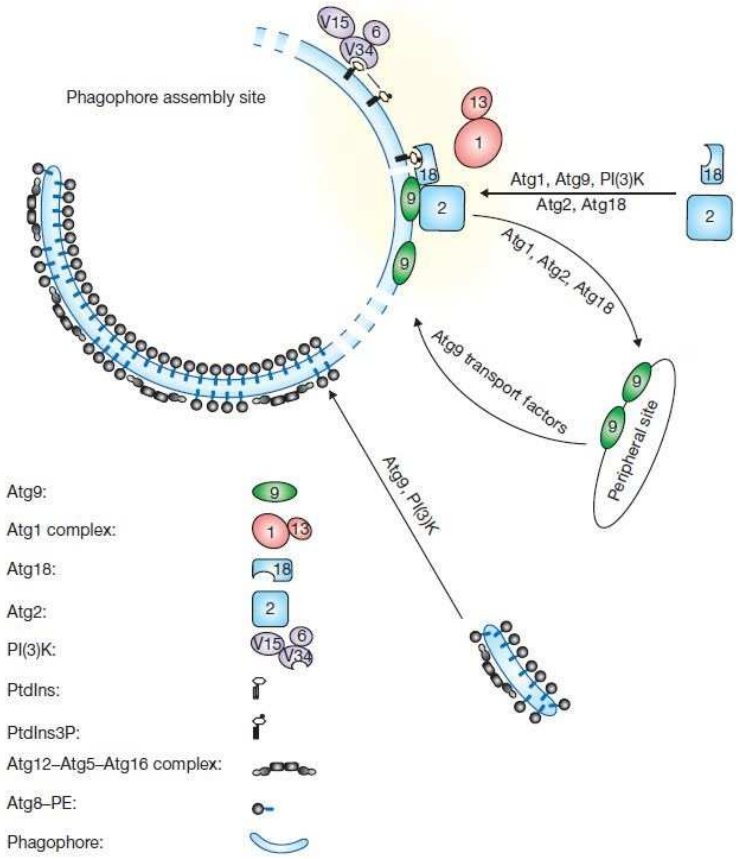

Figure 3. The phagophore assembly site
(Xie and Klionsky, 2007)

In yeast, Vps34 is a class III phosphatidylinositol 3- kinase (PtdIns3K) complex required for the formation of new autophagosomes. Atg6, Atg14 and the protein kinase Vps15 interact with Vps34 and form the autophagy-regulating macromolecular complex PI(3)K. This mechanism is conserved, formation of mammalian PtdIns3K includes hVps34, Beclin 1 (Atg6 homolog) and p150 (Vps15 homolog). The ortholog of Atg14 is called Atg14L or Barkor.

Beclin 1 binding proteins affect autophagosome formation. Proteins which induce autophagy include UVRAG (ultraviolet irradiation resistance-associated gene), ambra1- and bif-1, proteins which inhibit autophagy include Bcl-2 and Bcl-X_L. Antiapoptotic proteins such as Bcl-2 and Bcl-X_L bind to Beclin 1 and prevent the association with hVps34, which in turn lowers the PtdIns3K acivity and thereby inhibits autophagy (Yang and Klionsky, 2010b).

The serine-threonine kinase Atg1 is implicated in the initial steps of autophagosome formation in yeast. The kinase activity is essential for autophagy and regulates its magnitude (Xie and Klionsky, 2007). Several mammalian Atg1 proteins have been discovered, the most closely related and important are unc-51-like kinases (ULK). ULK1 and ULK2 exist in a macromolecular complex with mAtg13 (mammalian homolog of Atg13) and FIP200 (mammalian ortholog of Atg17). Under nutritient deprivation or other conditions that induce autophagy, mAtg13 and ULK1/2 are dephosphorylated and activated, which phosphorylates FIP200 to enhance autophagosome formation (Ravikumar et al., 2010).

Table 1 provides an overview of pivotal proteins in autophagosome formation:

Nucleation step	Mammalian Protein	Yeast Ortholog	Feature
ULK/Atg1 complex	ULK1, ULK2	Atg1	Protein kinase, phosphorylated by mTORC1
	Atg13	Atg13	Phosphorylated by mTORC1
	FIP200	-	Scaffold for ULK1/2 and Atg13
	Atg101 -	- Atg17, 29, 31	Interacts with Atg13 Interacts with Atg13
Class III PI3-kinase complex	Vps34	Vps34	PI3-kinase
	p150	Vps15	Myristoylated
	Beclin 1	Vps30/Atg6	BH3-only protein, interacts with Bcl-2
	Atg14	Atg14	Autophagy-specific subunit
	Ambra1	-	Interacts with Beclin 1
Others	Atg2	Atg2	Interacts with Atg18 in yeast
	Atg9	Atg9	Transmembrane protein
	WIPI1-4	Atg18	PI(3)P-binding proteins
	DFCP1	-	PI(3)P-binding ER protein
	VMP1	-	Transmembrane protein
Elongation Step			
Atg12-conjugation system	Atg12	Atg12	Ubiquitin-like, conjugates to Atg5
	Atg7	Atg7	E1-like enzyme
	Atg10	Atg10	E2-like enzyme
	Atg5	Atg5	Conjugated by Atg12
	Atg16L1	Atg16	Homodimer, interacts with Atg5
LC3/Atg8-conjugation system	LC3 (GATE-16, GABARAP)	Atg8	Ubiquitin-like, conjugates to PE
	Atg4A-D	Atg4	LC3/Atg8 C-terminal hydrolase, deconjugating enzyme
	Atg7	Atg7	E1-like enzyme
	Atg3	Atg3	E2-like enzyme

Table 1. Key Proteins in Mammalian Autophagosome Formation (Mizushima, Yoshimori and Levine, 2010)

3.3 Origin of the autophagosome membrane

Induction of the phagophore (or isolation membrane) from which autophagosomes arise is a central step in autophagy, yet the membrane source still remains unclear. Studies on the mammalian phagophore by fixation with reduced osmium in the 1980s suggested that the autophagosomal membrane was unique and generated de novo, since it is protein-poor. Several more recent research results indicate that while the phagophore is indeed unique, it arises from pre-existing intracellular compartments like the endoplasmatic reticulum (ER) or the Golgi complex (Ravikumar et al., 2010).

There is evidence that the ER is involved in phagophore formation. Atg14 is attached both to the ER and the phagophore, a mutated Atg14 unable to attach to the ER also loses its ability to induce autophagosomes (Tooze and Yoshimori, 2010). The discovery of a non-Atg protein named DFCP1 (double FYVE-domain-containing protein 1), which translocates from the Golgi to the omegasome (a subdomain of the ER) under nutrient deprivation, provided researchers with the opportunity to demonstrate the connection between the ER/omegasome and the phagophore membrane source. Findings by Matsunaga et al. (2010) show that a knockdown of Atg14L/Barkor results in disappearance of the DFCP1-positive omegasome. Atg14L is specific to the PI(3)K complex consisting of hVps34, p150 and Beclin 1.

A paper by Hailey et al. (2010) is not in accordance with the hypothesis that the ER is the sole source of the phagophore. The team used YFP-fusion proteins targeting different intracellular organelles (Golgi, TGN, plasma membrane, ER, mitochondria and the early endosomal system) in starved cells, and cyan fluorescent protein (CFP) fused to LC3. Only the marker for the outer mitochondrial membrane showed an sizable (80%) overlap with autophagosomes. They also found that the tail-anchor of a protein in the outer mitochondrial membrane is also located in autophagosome membranes. The authors hypothesize that under nutrition depletion, synthesis of PE (phosphatidylethanolamine) as the target of LC3 in the ER is restricted and autophagy may switch to transferring mitochondrial PE to the lysosomes.

In conclusion, the origin of the autophagosome membrane may be variable and is still a matter of debate.

3.4 Cargo recognition – selective autophagy

Autophagy has once been considered a nonselective “bulk” degradation process of cellular components. When cells are deprived of nutrients, this type of autophagy breaks cytosolic content down into metabolic building blocks to provide energy. In the last few years, a variety of selective autophagic forms were discovered. Selective autophagy is independent of nutrition status and specifically targets and degrades damaged organelles, misfolded proteins and various other cell contents. Several forms of selective autophagy have been described, inter alia the clearing of mitochondria (mitophagy), peroxisomes (pexophagy), ribosomes (ribophagy), lipids (macrolipophagy) and pathogens (xenophagy) (Youle and Narendra, 2011).

Besides autophagy, the ubiquitin-proteasome system (UPS) is the second major intracellular degradation pathway. The UPS conducts the fast and highly selective degradation of single short half-life proteins by linking them with ubiquitin chains, which serve as a recognition motif to 26S proteasomes (Kraft, Peter and Hofmann, 2010).
Previously autophagy and the UPS were thought to be independent of each other, yet evidence is emerging that these pathways are linked. Long-lived proteins can also be degraded by UPS, while short half-life proteins may be degraded by autophagy. Various proteins are degraded both by autophagy and the UPS. Inhibiting proteasomes upregulates autophagy, which may be a compensatory strategy (Ravikumar et al., 2010).

Observations by several authors indicate that ubiquitin-binding proteins p62, SQSTM1 (sequestosome 1) and NBR1 (neighbour of Brca1 gene) play a role in connecting autophagy and UPS. If the capacity of 26S proteasomes to dispose misfolded proteins is exceeded, these proteins polymerize and form structures called inclusion bodies. The autophagic degradation of inclusion bodies depends on the presence of p62 and its interaction with LC3 and ubiquitin (Ub). Ub-containing inclusion bodies also hold LC3 (Kraft, Peter and Hofmann, 2010). p62 acts as an autophagic adaptor between ubiquinated substrates and the autophagic machinery.

Selective autophagy is based on the ability of autophagic adaptors to specifically recognize substrates. Human p62 protein is 440 amino acids long and contains, among other domains, a N-terminal PB1 domain, LIR (LC3-interacting region) and a C-terminal UBA (Ub-associated) domain. The PB1 domain enables oligomerization of p62 and NBR1, homopolymerization of p62 and interaction with several protein kinases. NBR1 is 966-amino acids long and has a similar domain structure, both proteins connect to LC3 via their LIR motif, which is necessary for autophagic degradation (Johansen and Lamark, 2011).

3.5 Autophagosome-lysosome fusion

The autolysosome is generated by fusion of the immature autophagosome with endosomes and lysosomes. Insight into how this fusion is controlled remains fragmentary and an unresolved issue.
Multi-vesicular bodies (MVB), generated from early endosomes, are required for autophagosome maturation. Mutation of proteins which enable MVB generation (ESCRT, SNARE, Rab7, class C Vps) block maturation (Ravikumar et al., 2010).
COPI, a coatomer protein complex known for coating vesicles transporting proteins from the Golgi to the ER, is also involved in maintaining endosomal/lysosomal function. Razi, Chan and Tooze (2009) demonstrated that a lack of coatomer subunits leads to an accumulation of autophagosomes, since the loss of COPI blocks transport into early endosomes and impairs autophagosome maturation.
Findings by Lee et al. (2010) show that the ubiquitin-binding protein deacetylase, histone deacetylase-6 (HDAC6) plays a major role in nutrient-independent basal autophagy. HDAC6 is not required for autophagy activation or autophagosome formation, but in the absence of HDAC6, autophagosomes accumulate due to an autophagosome-lysosome fusion deficiency. Interestingly, HDAC6 is not required for starvation-induced autophagy, which indicates that these two autophagic modes are fundamentally different.
Settembre et al. (2011), studying transcription factor EB (TFEB), found that TFEB is not only a master gene for lysosomal biogenesis, but also regulates autophagy. TFEB co-regulates the expression of autophagy and lysosomal genes.

4. Signalling pathways

4.1 PI3K/mTOR pathway

mTOR, the mammalian ortholog of yeast protein kinase TOR, is the master regulator of mammalian autophagy and the most studied pathway. TOR is a highly conserved serine-threonine protein kinase and an important signaling molecule. It exist in two functional complexes which are conserved from yeast to mammals: TORC1 and TORC2. TOR complex 1 (TORC1) is rapamycin-sensitive and negatively regulates autophagy, TORC2 is no direct autophagy regulator (Ravikumar et al., 2010).
mTORC1 consists of mTOR, raptor (regulatory associated protein of mTOR), mLST8 (MTOR associated protein, LST8 homolog) and PRAS40 (proline-rich Akt substrate of 40 kDa). mTORC1 is a sensor of nutrient signals, energy status (AMP/ATP), amino acids, glucose and various forms of stress.

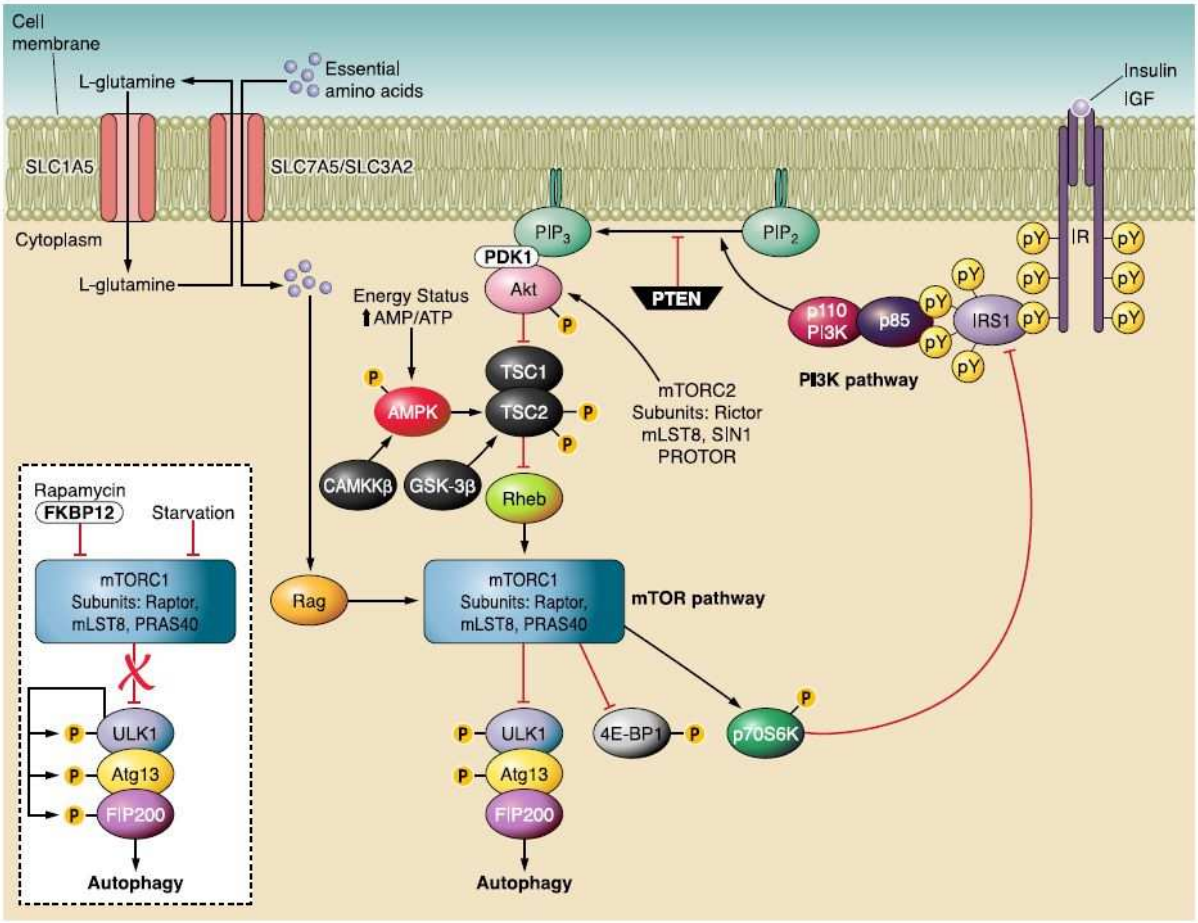

Figure 4. The PI3K-mTOR pathway regulating autophagy
(Ravikumar et al., 2010)

Inhibition of mTORC1 by rapamycin reduces phosphorylation of translation initiation factor 4E-BP1 (4E binding protein-1) and p70S6K (ribosomal protein S6 kinase-1), which both are cell growth regulators.
The tuberous sclerosis complex 1 and 2 (TSC1/TSC2) is a stable heterodimer which senses upstream inputs from several kinases, including Akt (RAC-alpha serine/threonin-protein kinase). Phosphorylation of TSC2 disaggregates the complex and activates mTOR.
AMPK (AMP-activated protein kinase), a sensor of cellular bioenergetical changes in the ATP/AMP ratio, is activated by a decreased ratio and in turn phosphorylates TSC2, connecting mTORC1 with energy factor signaling. Amino acids suppress autophagy via Rag GTPase in nutrient response.
The activation of receptors at the cell surface by insulin or growth factors leads to the autophosphorylation of receptor tyrosine kinases, activating class Ia PI3K (Phosphatidylinositol 3-kinase) and its p110 catalytic subunit. PI3K turns PIP_2 (Phosphatidylinositol 4,5-bisphosphate) into PIP_3 (Phosphatidylinositol (3,4,5)-triphosphate), which recruits Akt to the plasma membrane, where it is activated by phosphorylation. Akt inhibits the activity of TSC1/TSC2.
The Atg13-ULK1-FIP200 complex signals to the autophagic machinery downstream of mTORC1 (Yang and Klionsky, 2010b; Ravikumar et al., 2010).

4.2. mTOR-independent pathways

Autophagy is negatively regulated by IMPase (inositol monophosphatase), which lowers intracellular inositol and IP_3 (myoinositol-1,4,5-triphosphat) levels. Activation of PLC (phospholipase C) hydrolyzes PIP_2 to DAG (diacylglycerol) and IP_3, which in turn binds to ER IP_3 receptors, prompting the release of Ca^{2+}. IP_3 is degraded to IP_1 (inositol monophosphate) and hydrolyzed into free inositol by IMPase. ER IP_3 receptors also bind Beclin-1, negatively regulating autophagy.
Psychiatric drugs like lithium or carbamazepine positively regulate autophagy via this pathway. By competing with the cofactor Mg^{2+}, Li^{+} inhibits IMPase, interferes with inositol recycling and inhibits the phosphoinositol pathway (Rubinsztein, Marino and Kroemer, 2011).

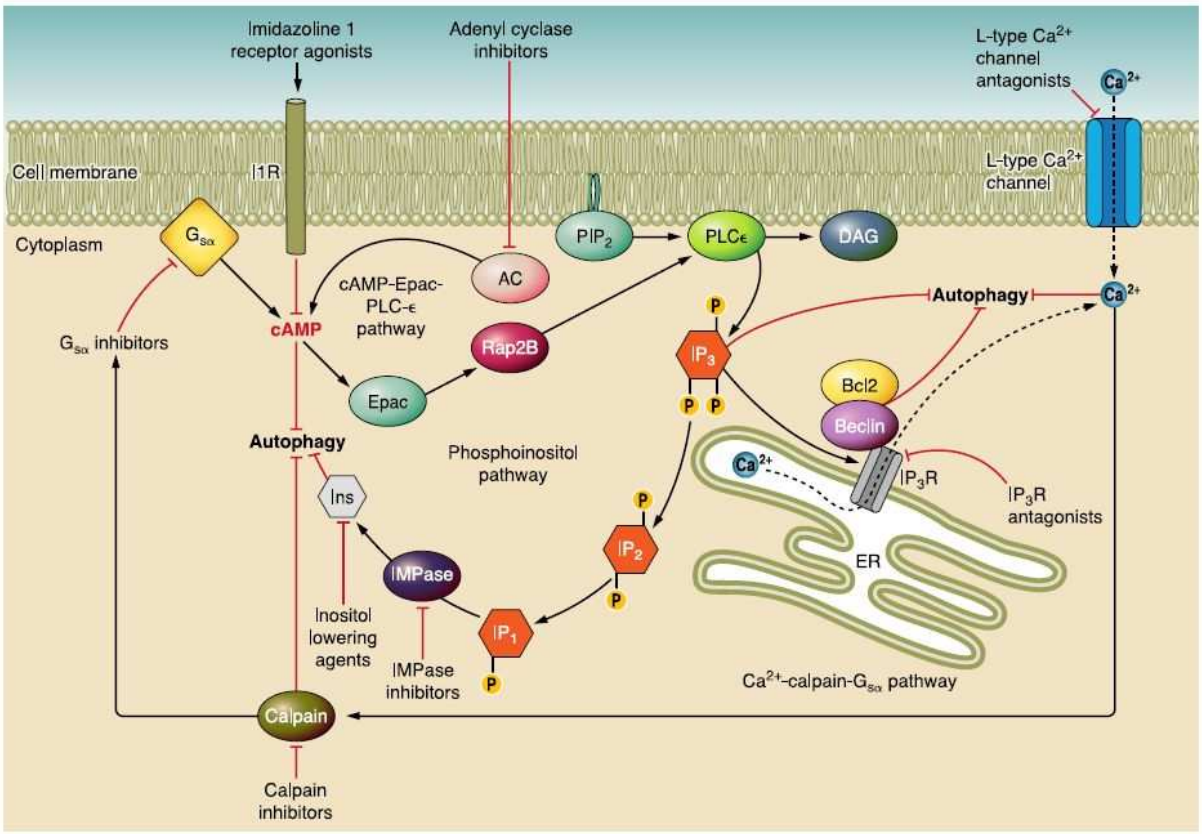

Figure 5. mTOR-independent pathways regulating mammalian autophagy
(Ravikumar et al., 2010)

Cyclic adenosine monophosphate (cAMP) is central to a different mTOR-independent autophagic pathway. The intracellular cAMP level negatively regulates autophagy via its target Epac (guanine nucleotide exchange factor). Epac activates Rap2B, a protein which in turn activates PLC. Pharmaceuticals like clonidine, a imidazoline-1 receptor antagonist, depress cAMP levels and induce autophagy.

Another mTOR-independent pathway is mediated by the intracytosolic Ca^{2+} level. As has already been mentioned, IP_3 binding to its ER receptors (IP_3R) opens Ca^{2+} stores. Inhibition of IP_3R or chemical agents like Ca^{2+} channel antagonists which decrease Ca^{2+} levels induce autophagy, drugs that release Ca^{2+} from ER stores inhibit autophagy. Calpain, a family of Ca^{2+}-dependent cysteine proteases, acts downstream of free cytosolic Ca^{2+}. Calpain activation inhibits autophagy, genetic knockdown of calpain induces autophagy (Ravikumar et al., 2010).

5. Aging, health and disease

5.1 Aging

As reviewed by Rubinsztein, Marino and Kroemer in 2011, there are various reports and hints that link autophagy and aging. Autophagy inhibition leads to premature aging or changes in mammalian tissues similar to aging, increased autophagy extends longevity and reduces cell death, at least in model organisms.

During aging, cellular housekeeping mechanisms decline and fail to clear damaged intracellular macromolecules like organelles, proteins or lipids. Autophagy diminishes with aging, ATG proteins have less expression in aged tissues (Ravikumar et al., 2010). For example, in normal human brain aging Atg5, Atg7 and Beclin 1 are downregulated, as are ULK1, Beclin 1 and LC3 in osteoarthritis.

Autophagy inhibition by reducing the activity or loss-of-function mutations of TOR/mTOR, essential ATG proteins, vps-34, FIP200, Beclin 1 or other components of the autophagic pathway contributes to the aging phenotype and age-associated pathologies (Table 2) (Rubinsztein, Marino and Kroemer, 2011).

Caloric restriction (CR) – reduced calorie diet without underfeeding – extends the life span of most animals so far tested, and is the main physiological autophagy inducer through energy sensors AMPK and Sirtuin 1 (SIRT1). Inhibiting autophagy negates the longevity gains, as does knocking out atg genes after administration of rapamycin.
Resveratrol, a natural polyphenol, prolongs life span by activating SIRT1, which deacetylates several atg gene products and various transcription factors like p53, FOXO1 and PGC1α.
Spermidine is a polyamine acting as a histone acetylase inhibitor. Spermidine upregulates the expression of atg genes, stimulates deacetylation reactions of cytoplasmic proteins, reduces histone acetylation and induces autophagy. The life-span prolonging effect of spermidine is lost when atg genes are knocked out or knocked down (Table 3) (Rubinsztein, Marino and Kroemer, 2011).

Autophagy Deficiency and Genotype	Phenotype	Disease Relevance
Heterozygous knockout of Beclin 1	Enhanced inflammation, steatohepatitis, enhanced frequency of hepatocellular carcinomas, lung adenocarcinomas, and lymphomas in aging mice; enhanced accumulation of intraneuronal amyloid ß upon transgenic expression of human amyloid precursor protein	Aging-related malignancies; neurodegeneration
Central nervous system-specific knockout of Atg5 or Atg7	Progressive accumulation of ubiquitinylated proteins as inclusion bodies in neurons, followed by cortical and cerebellar neuronal loss and premature death	Neurodegeneration
Purkinje cell-specific knockout of Atg5 or Atg7	Axonal dystrophy and degeneration of axon terminals; subsequent Purkinje cell death and cerebellar ataxia	
Central nervous system-specific knockout of FIP200	Accumulation of ubiquitinylated proteins as inclusion bodies in Purkinje neurons, cerebellar cell loss, and cortical spongiosis	
General knockout of HDAC6	Intracellular ubiquitin^{+} aggregates in the brain from 6 months of age	Neurodegeneration
General knockout of PINK1	Selective mitochondrial defect (complex I + II) in the striatum	Parkinson's disease
Dynein mutations (fly and mouse models)	Premature aggregate formation of mutant huntingtin with increased autophagosome formation and impaired autophagosome-lysosome fusion	Huntington's disease, motor neuron diseases
Liver- and spleen-specific interferon-γ-inducible knockout of Atg7	Hepatomegaly, accumulation of peroxisomes, deformed mitochondria and ubiquitin-positive inclusions in hepatocytes, hepatocyte death (increased serum level of alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase); accumulation of p62/STQM and consequent NRF2 activation	Hepatopathy
Hepatocyte-specific knockout of Atg7	Lipid droplets containing triglycerides and cholesterol accumulate in liver cells; ER stress in hepatocytes and insulin resistance	Metabolic syndrome, steatohepatitis
Skeleton-muscle specific knockout of Atg7	Muscle atrophy and age-dependent decrease in force; accumulation of abnormal mitochondria, sarcoplasmic reticulum distension, disorganization of sarcomere; enhanced muscle loss after fasting or denervation	Sarcopenia
Islet ß cell-specific knockout of Atg7	Accumulation of ubiquitinated protein aggregates colocalized with p62, mitochondrial swelling, endoplasmic reticulum distension, and vacuolar changes; degeneration of islets and impaired glucose tolerance with reduced insulin secretion; enhanced susceptibility to high-fat diet-induced diabetes; this phenotype can be improved by feeding the antioxidant N-acetylcysteine	Insulin-dependent diabetes
Cardiomycyte-specific constitutive knockout of Atg5	No cardiac phenotype in baseline conditions but enhanced susceptibility to acute pressure overload-induced cardiac dysfunction and left ventricular dilatation	Dilated cardiopathy
Podocyte-spccific [*sic*] constitutive knockout of Atg5	Spontaneous age-dependent late onset glomerulosclerosis with accumulation of oxidized and ubiquitinylated proteins, compensatory proteasome activation, lipofusin, damaged mitochondria, ER stress, albuminuria, podocyte loss	Glomerulosclerosis
Atg5$^{flox/flox}$;Podocin-Cre^{+}	Enhanced susceptibility to glomerulopathy induced by injections of doxorubicin or puromycin aminonucleoside	

Table 2. **Selected Aging-Related Phenotypes of Autophagy-Deficient Mice** (Rubinsztein, Marino and Kroemer, 2011)

Longevity-increasing/Anti-aging Manipulations	Phenotype	Relationship to Autophagy
daf-2 (Insulin/IGF-1 receptor) loss-of-function mutant in C. elegans	Enhanced autophagy and life span	Abolishment of longevity phenotype in animals with loss-of-function mutations of *bec-1* or upon *atg-7* or *atg-12* RNAi
Dietary restriction mutant eat-2 (ad1113) in *C. elegans*.		Loss of longevity phenotype after *bec-1* and *atg7* RNAi
Calcineurin (cnb-1) null mutant (jh103) in *C. elegans*		Loss of longevity phenotype after *bec-1* and *atg7* RNAi
Administration of rapamycin to *C. elegans*		Loss-of-function mutations of Atg1 or Atg7 abolish life span extension
Administration of spermidine to *C. elegans*		Loss of longevity phenotype after *bec-1* RNAi
Administration of resveratrol to *C. elegans*		Loss of longevity phenotype after *bec-1* RNAi
Transgenic expression of Atg8a in the brain of *D. melanogaster*	Counteracts the age-associated loss of Atg8a expression; increased life span (up to 56% in females), reduced accumulation of insoluble ubiquitinated proteins and carbonylated proteins, increased resistance against H_20_2	Direct increase in autophagy within neurons
Transgenic expression of FOXO in the muscles of D. *melanogaster*	Improved proteostasis and muscle function during aging; enhanced longevity	Enhanced muscle autophagy; knockdown of Atg7 enhances the deposition of polyubiquitinated protein aggregates
Administration of rapamycin to *Drosophila*	Enhanced autophagy and life span	Loss of longevity phenotype after *atg-5* RNAi
Administration of spermidine to *Drosophila*	Enhanced autophagy and life span	Loss-of-function mutations of *atg-7* abolish life span extension
Increase of LAMP-2A abundance by means of an inducible, hepatocyte-specific transgene in mice; LAMP2a-Alb-Tet-off	Reduced abundance of oxidized proteins, polyubiquinated protein aggregates, and $TUNEL^+$ cells	Restoration of chaperone-mediated autophagy and macroautophagy in livers from aged animals
Cardiomyocyte-specific expression of a dominant-negative PI3Kα (p110α)	Enhanced autophagy, reduced lipofuscin levels in the heart, and increased longevity	Same as above
Knockin mutation of huntingtin, causing the deletion of the polyglutamine stretch (ΔQ); $Htt^{\Delta Q/\Delta Q}$	Increased longevity of mice; the ΔQ Htt protein induces autophagy in vitro	Same as above
Administration of rapamycin to mice	Extension of maximum life span by up to 14% in males and females accompanied by mTOR inhibition	Same as above

Table 3. **Associations between Autophagy and Aging in Animals** (Rubinsztein, Marino and Kroemer, 2011)

5.2 Cancer

In 1999, Levine's group (Liang et al., 1999) published the first report linking *beclin 1*, a mammalian gene with a role in autophagy, with the inhibition of tumorigenesis. Originally, Beclin 1 was isolated as a Bcl-2 (B-cell lymphoma 2) interacting coiled coil protein. Using gene-transfer techniques, the research group found that *beclin 1* promotes autophagy in human MCF7 breast carcinoma cells, and inhibits MCF7 cellular proliferation. Beclin 1 also inhibits tumorigenesis in nude mice.
Monoallelic deletion of *beclin 1* on human chromosome locus 17q21 occurs in 40-75% of human ovarian, prostate and breast cancers. The expression of Beclin 1 is decreased in carcinomas. Out of 11 human breast carcinoma cells, Liang et al. found detectable levels of Beclin 1 in only three of them, despite higher levels of total protein (Liang et al., 1999; Yang and Klionsky, 2010a).
Observations by Qu, while working in Levine's laboratory in 2003, suggested that the genetic disruption of autophagy is an important mechanism of oncogenesis. The heterozygous loss of *beclin 1* in targeted mutant mouse models led to reduced autophagy and increased spontaneous tumorigenesis. Only 1% of *beclin* $1^{+/+}$ mice had palpable tumors, compared to 15% of their of *beclin* $1^{+/-}$ littermates. Thus, beclin 1 is a haploinsufficient tumor suppressor (Qu et al., 2003).

However, autophagy may also promote the survival of tumor cells. Metabolic stress like hypoxia and nutrient deprivation leads to an upregulation of autophagy in an self-preservation attempt of malignant cells. In agreement with this, pharmacological inhibition of autophagy aids therapy-induced apoptotic tumor cell death and enhances the cytotoxicity of chemotherapy agents (Ravikumar et al., 2010). Findings by Amaravadi et al. (2007) show that treatment of tumor cells with chloroquine (CQ), an autophagic inhibitor, and cytotoxical agents significantly enhanced tumor regression and delayed tumor recurrence in mice.

5.3 Neurodegeneration

Several studies provided evidence that autophagy induction is a therapeutic strategy and beneficial physiological response in a variety of adult-onset human neurodegenerative diseases like Alzheimer's, Parkinson's or Huntington's disease. The accumulation of abnormal, pathological aggregates within neurons and other cell types is a common feature of these neurodegenerative diseases. The mutant huntingtin protein is the constituent of such aggregates in Huntington's disease, akin to the intraneuronal tau protein and amyloid beta (Aβ) deposits in Alzheimer's disease (AD). In general, basal autophagy prevents accumulations of soluble, cytosolic proteins, furthermore, selective autophagy may clear these aggregates (Ravikumar et al., 2010; Yang and Klionsky, 2010a).

Yu et al. (2005) examined the occurrence of autophagosomes in neuronal cell lines and in the brain at early AD. The authors observed elevated levels of LC3-II in mice brain at early, prepathological stages of AD, indicating that autophagy is an early response in the disease process. Komatsu et al. (2006), examining the relationship between neuronal pathology and autophagy, crossed *Atg7*-conditional knockout mice (*Atg7*$^{flox/flox}$) with transgenic mice (nestin-*Cre)* to produce mice with central nervous *Atg7* deficiency.

In contrast to their control (*Atg7*$^{flox/+}$ nestin-*Cre*) littermates, *Atg7*$^{flox/flox}$ nestin-*Cre* mice displayed motor and behavioural deficits, growth retardation and the survival rate diminished significantly four weeks after birth, and was zero within 28 weeks. Inclusion bodies of polyubiquinated proteins, which increased in size and number with ageing, accumulated in autophagy-deficient neurons. These results indicate that autophagy is essential for the function of the central nervous system.

5.4 Immunity

In groundbreaking research, Yoshimori's (Nakagawa et al., 2004) as well as Deretic's and Colombo's group (Gutierrez, Master, Singh, Taylor, Colombo and Deretic, 2004) reported that autophagy is a defence mechanism against invading bacterial pathogens *Streptococcus pyogenes* and *Mycobacterium tuberculosis*, respectively.
Gutierrez et al. observed the acidification and colocalization of mycobacterial phagosomes with LC3 after physiological or pharmaceutical induction of autophagy. Rapamycin treatment reduced mycobacterial viability and enhanced phagosomal maturation in a dose-dependent manner. The assumption that *M. tuberculosis* phagosomes are diverted to a compartment with autophagic characteristics has been confirmed by electron microscopy.
A similar mechanism was reported by Nakagawa et al. for *Streptococcus pyogenes* (GAS). GAS was trapped in autophagosomal compartments and killed after fusion of these with lysosomes. GAS invasion induced autophagy, since the amount of LC3-II increased after infection. No induction of LC3-II was observed in autophagy-deficient Atg5$^{-/-}$ cells, and GAS multiplied.

The sequestration of bacteria in autophagosomes as a key component of host defense evolutionary favoured the appearance of mechanisms to evade or subvert this response. (Ravikumar et al., 2010).
Ogawa, Yoshimori, Suzuki, Sagara, Mizusima, and Sasakawa (2005) noted the ability of *Shigella*, an invasive bacteria closely related to *E. Coli*, to escape autophagy. *Shigella* protein VirG induces autophagy by binding to Atg5, yet is competitively inhibited by IcsB, an effector secreted by its type III secretion system. As a result, the interaction of IcsB with VirG inhibits autophagy in the vicinity of the bacterial surface.
The first report of a microbial virulence factor directly antagonizing the autophagic machinery came from Oredahl et al. in 2005. Herpes simplex virus type 1 (HSV-1) encodes the neurovirulence protein ICP34.5, which directly interacts with the mammalian autophagy protein Beclin 1. Beclin 1 is part of the autophagy regulating macromolecular complex PI(3)K (for details, see chapter 3). Beclin 1-binding-deficient mutant HSV-1 fails to inhibit autophagy and displays diminished neurovirulence.

6. Conclusions

As outlined in this thesis, our understanding of mammalian autophagy has extensively increased in the last decade. Since autophagy is involved in a wide range of physiological processes and diseases, knowledge of its basic biological mechanisms is vital. Currently available techniques and methods to monitor and modulate autophagy in mammalian cells are limited or non-existent, in particular with regard to measuring the autophagic flux in living humans. Many outstanding questions still need to be answered. For instance, the membrane source for autophagosome formation still remains inscrutable, as does the autophagic machinery with regards to signal input and magnitude regulation of autophagy.

While our current knowledge of mammalian autophagy may still only represent the tip of the iceberg, it is tempting to speculate that in the future we may be able to manipulate autophagy to prolong life span and fight disease.

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