Chapter 1

General Introduction
Chapter 1

1. Macroautophagy
1.1 The processes of macroautophagy
Cellular and organismal homeostasis is maintained through the balance between the synthesis and degradation of the cellular components, including proteins, nucleic acids, lipids. While the ubiquitin-proteasome pathway mainly mediates the exclusive degradation of proteins, autophagic processes allow the turnover of practically all cellular constituents. Autophagy is thus one of the major catabolic pathways in eukaryotes and cells make use of autophagy to adapt to environmental stresses and maintain their homeostasis. Three major types of autophagy have been described: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). The next chapters will describe in detail macroautophagy, also referred hereafter simply as autophagy.

The hallmark of autophagy is autophagosomes. Induction of autophagy leads to the de novo generation of a cup-shaped membrane called isolation membrane (IM) or phagophore. In yeast the IM is formed at a perivacuolar subcellular location called the phagophore assembly site or preautophagosomal structure (PAS). The origin of the autophagosomal membranes is still a matter of debate and it will be discussed in detail in chapter 1. Cytoplasmic content targeted to degradation such as organelles or proteinaceous aggregates are enwrapped by the elongating IM, which eventually seals at its extremities, leading to the formation of a double-membrane autophagosome that contains the cargo (Figure 1). Subsequently, the outer membrane of the autophagosome fuses with the lysosome (in mammals) or the vacuole (in plants and in yeast) leading to the release of the cargo in the interior of these organelles, where it is turned over by resident hydrolases (Figure 1). The metabolites resulting from the degradation of the autophagosomal cargoes are then exported into the cytoplasm to be reused by the cell.

Autophagy has been known for years as a process of bulk degradation of cytoplasmic components, but recent evidences have revealed that autophagosomes can selectively sequester and turnover specific intracellular structures or invading microbes. Numerous organelles can
be selectively degraded through autophagy, including the endoplasmic reticulum (ER), mitochondria, peroxisomes, lipid droplets, nucleus, pathogens, cytoplasmic aggregates, ribosomes and lysosomes through pathways defined as reticulopagy or ERpagy, mitophagy, pexophagy, lipophagy, nucleophagy, aggrephagy, ribophagy and lysosomes, respectively.1,2.

Figure 1. Schematic representation of the mechanism of autophagosome formation (in yeast). Under autophagy induction conditions, a cup-shaped membrane called isolation membrane or phagophores is formed in the cytoplasm. The phagophore elongates and during this expansion it sequesters cytoplasmic material including organelles and protein aggregates. The expanded phagophore seals leading to the formation of a double membrane vesicle known as the autophagosome. The autophagosome then fuses with the outer vacuolar membrane releasing its cargo into the vacuolar lumen where it is degraded by resident hydrolases. The hydrolysis products, i.e. amino acids, sugars, lipids, nucleotides..., are transported into the cytoplasm where they are used as new building blocks for macromolecules or as a source of energy.

1.2 Yeast as a model for the study of the mechanism of autophagy
The term autophagy was first used more than 50 years ago to define a process that delivers cell cytoplasmic components and organelles into the lysosome for degradation.3,4 The molecular principles underlying this pathway, however, remained mysterious for a long time. In the 1990’s, the existence of autophagy in the budding yeast Saccharomyces cerevisiae was demonstrated exploiting vacuolar proteases-deficient cells.5 In particular, large vesicles containing cytoplasmic material, named autophagic bodies, were detected accumulating in the vacuole of these yeast strains upon nutrient starvation.5 Based on this discovery, Tsukada and co-workers performed a genetic screen aimed to isolate mutants that were defective for starvation-induced autophagy.6 Almost
in parallel, genetic screens performed in yeast by others laboratories identified the same and additional genes involved in autophagy, which collectively were later called autophagy-related (ATG)\(^7-10\). As the ATG genes are highly conserved among eukaryotes and yeast can be easily genetically manipulated, this organism became an important model system to investigate the regulation, the mechanism and the functions of autophagy in eukaryotes. 

Another major experimental advantage of yeast is that it possesses a biosynthetic selective type of autophagy called the cytosol-to-vacuole targeting (Cvt) pathway, which has enormously facilitated the study of this and other selective forms of autophagy. The Cvt pathway operates under nutrient-rich, growing conditions and mediates the transport into the vacuole of oligomers, also known as the Ape1 complex, formed by the vacuolar hydrolases aminopeptidase 1 (Ape1), aminopeptidase 4 (Ape4) and α-mannosidase (Ams1), and the Ty1 transposome\(^2,11\).

### 1.3 The autophagy machinery

From all the 41 ATG genes identified so far in yeast, 16 of them compose the conserved core machinery that mediates the autophagosome formation in all the different types of non-selective and selective autophagy\(^2,12\). Most of these Atg proteins are cytoplasmic and associate together at the PAS upon autophagy induction through what appears to be an hierarchical mechanism\(^12,13\). Although the exact molecular function of this machinery in the formation, elongation and fusion of the autophagosome is far from being clarified, these genes have been classified into five main functional groups mostly based on physical interactions: (1) the Atg1/ULK kinase complex, (2) the phosphatidylinositol 3-kinase (PI3K) complex, (3) the Atg9 trafficking system and (4-5) the Atg12 and Atg8 ubiquitin-like conjugation systems (Figure 2).
**Figure 2. Representation of the core Atg protein functional groups.** These groups have been classified through physical and genetic interactions as: (1) the Atg1/ULK kinase complex, (2) the phosphatidylinositol 3-kinase (PI3K) complex, (3) the Atg9 trafficking system and (4-5) the Atg12 and Atg8 ubiquitin-like conjugation systems.

1.3.a. **Atg1/ULK1 kinase complex**

Atg1/ULK1 is a serine/threonine protein kinase, which has a key role in the regulation of autophagy induction\textsuperscript{14}. In yeast, it is part of the Atg1/ULK complex, which consists of two sub-complexes: the constitutive Atg17-Atg31-Atg29 sub-complex and Atg1-Atg13 sub-complex, which is formed upon starvation and binds Atg17-Atg31-Atg29 through Atg13 leading to the activation of the Atg1 kinase activity (Figure 3)\textsuperscript{15,16}. Atg1 and mammalian Unc51-like kinase1 (ULK1) possess a serine-threonine kinase and two microtubule interacting and transport (MIT) domains at N-terminus and C-terminus, respectively, which are domains conserved among eukaryotes\textsuperscript{17}. The MIT domains mediate Atg1/ULK1 interaction with Atg13 and membranes\textsuperscript{18–20}. In presence of nutrients, the target of rapamycin
complex 1 (TORC1) phosphorylates Atg13 keeping this protein apart from Atg1. Under starvation conditions, inactivation of TORC1 leads to the dephosphorylation of Atg13 promoting its association with Atg1 and Atg17 (Figure 3). A model for the activation of the Atg1/ULK complex has been proposed. That is, Atg17, which has a crescent-like shape, in its trimeric complex Atg17-Atg31-Atg29 complex dimerizes at the PAS, and this promote the recruitment to this location of the Atg1-Atg13 dimer. The formation of a decameric complex induces in turn Atg1 kinase activity and the subsequent recruitment of the rest Atg proteins to the PAS, which are necessary for the formation of an autophagosome.

Atg11, a coiled-coil protein, is required for the selective recruitment of cargo proteins to autophagosomes, as e.g. the Ape1 complex, mitochondria, peroxisomes, ER and nucleus but it is not required for non-selective bulk autophagy. The study of the Cvt pathway has provided insights into how Atg11 could coordinate cargo selection and autophagosome formation through the modulation of the Atg1/ULK complex. Binding of the Ape1 complex to the specific autophagy receptor Atg19, promotes the subsequent interaction of Atg19 C-terminus with Atg11, which in turn binds to Atg1 and Atg17. Atg11-Atg1 interaction is essential to activate Atg1 kinase activity, which triggers the formation of an autophagosome around the cargo.

In mammals, ULK1 or ULK2, two redundant Atg1 homologues, interact with mATG13 and focal adhesion kinase family-interacting protein of 200 kDa (FIP200), which are the counterparts of Atg13 and Atg17, respectively. The mammalian ULK complex also includes the non-conserved component Atg101 but no homolog proteins of Atg29 and Atg31.

Atg1 and ULK1 interact also with Atg8/LC3 through a LC3-interacting region (LIR) motif. In yeast, this binding seems to promote the vacuolar degradation of Atg1-Atg13 complex, which could work as a feedback regulatory step of autophagy. Similarly, it was also shown that ATG13 and FIP200 interact with LC3 in mammals reinforcing this notion.
1.3.b. Phosphatidylinositol 3-kinase complex

PI3K phosphatidylinositol 3-kinase (PI3K) complex phosphorylates the 3-hydroxyl group of the inositol ring from three species of phosphatidylinositol (PtdIns) lipid substrates: PtdIns, PtdIns-4-phosphate, and PtdIns-4,5-bisphosphate. The generated 3-phospho-inositol phosphates promote the recruitment of diverse effector proteins that bind these lipids through specific phosphoinositide-binding motifs, e.g. the pleckstrin homology (PH) domain, the phox homology (PX) domain, and the FYVE domain. The coordinated activity with 3-phosphoinositide phosphatases and the low phosphoinositide-binding affinity of these domains permit highly reversible effector localization and cellular responses. These 3-phosphoinositides can be localized to diverse cellular membranes and their phosphorylation status can be rapidly reversed, making them key regulators of diverse processes at intracellular membranes, including signaling cascades and intracellular membrane trafficking events.

Figure 3. The Atg1/ULK1 kinase complex and its regulation in response to amino acid levels. Target of rapamycin complex 1 (TORC1) is active under nutrient rich conditions, phosphorylating Atg13 and keeping Atg13 disassociated from the Atg1 kinase. Upon amino acid deprivation (or rapamycin treatment), TORC1 is inactivated and Atg13 is concomitantly dephosphorylated. Dephosphorylated Atg13 binds Atg1 and the formed dimer interacts with the Atg17, Atg29 and Atg31 trimer present at the PAS, leading to the formation of the Atg1 complex and activating the kinase activity of Atg1. Atg1 activity is thought to be at the core of autophagosome formation in response to stimuli.
All PI3K bear a PI3K signature motif, composed of a C2 domain, important for the interaction with membranes, a helical domain and the catalytic kinase domain\textsuperscript{44}. The PI3K family is subdivided into three classes, i.e. class I, II and III, based on the conserved domains outside the signature motif, the association with regulatory subunits, and their preferred lipid substrate\textsuperscript{48}. Each class has multiple cellular roles through the regulation of distinct phosphoinositide pools. Class I PI3K mainly produce PtdIns-3,4,5-triphosphate and indirectly PtdIns-3,4-biphosphate, class II generates PtdIns-3,4-biphosphate and PtdIns-3-phosphate (PtdIns-3-P), and class III exclusively forms PtdIns-3-P. A single orthologue of each class can be found in \textit{Caenorhabditis elegans} and \textit{Drosophila melanogaster}, while yeast contains only one class III PI3K\textsuperscript{44,45,49}.

Class III PI3K are conserved among all eukaryotes and has a unique member, the vacuolar protein sorting 34 (Vps34), which in mammals is also known as PIK3C3\textsuperscript{50}. Vps34 forms different protein complexes involved in the synthesis of PtdIns-3-P pools at distinct intracellular membranes\textsuperscript{50}. In yeast, Vps34 is part of at least two distinct complexes, i.e. complex I and II, which have different subcellular localizations and functions (Figure 4)\textsuperscript{48}. Complex I PI3K is assembled at the PAS, where it synthesizes PtdIns-3-P, which is essential for the initiation and progression of autophagosome biogenesis, while complex II PI3K is present on endosomes and it is involved in membrane trafficking to and from these organelles\textsuperscript{48}. Complex I is composed by the Vps34/hVPS34, Vps15/p150, Atg6/BECLIN1, Atg14/ATG14L/BARKOR and Atg38/NRBF2, while complex II is formed by Vps34/hVPS34, Vps15/p150, Atg6/BECLIN1 and Vps38/UVRAG (Figure 4)\textsuperscript{48,51,52}.

PtdIns-3-P has been shown to be required for autophagy in all organisms. Autophagy is completely blocked in yeast lacking Vps34, as well as in cells expressing a Vps34 kinase-dead mutant\textsuperscript{53,54}. Similar results have been obtained in \textit{Drosophila}\textsuperscript{55}. Autophagy was also blocked when inhibiting the Vps34 kinase activity in different mammalian cell types treated with Vps34 inhibitors such as wortmannin and 3-methyladenine\textsuperscript{54,56,57}.
The work of Axe and collaborators has allowed a better understanding on how PtdIns-3-P is involved in the formation of the autophagosomes in mammals\textsuperscript{58}. They showed that the formation of ER derived omega-like structures, which they termed omegasomes, establishes a platform for the biogenesis of the mammalian phagophores. PtdIns-3-P, together with ULK1, are required for the formation of the omegasome, and PtdIns-3-P-enriched membranes subsequently promote the recruitment of WIPI2 and DFCP1, and later the ATG12-ATG5-ATG16 complex and LC3-PE to further expand the IM into autophagosomes\textsuperscript{43,58,59}. The distribution of PtdIns-3-P in the yeast and mammal autophagosome was shown to be different, with a mainly luminal distribution in yeast and an exclusive cytoplasmic one in mammals. These could be due to partial differences in the autophagy mechanism among these organisms\textsuperscript{60}. Recently, it was shown that myotubularin (MTMR) phosphatases, which convert PtdIns-3-P back to PtdIns, are also important for autophagy progression. Both Jumpy/MTMR14 and MTMR3 were shown to negatively regulate autophagy at early stages\textsuperscript{61,62}. Ymr1, the only yeast MTMR phosphatase, is also essential for normal progression of autophagy and mechanistically, it appears to promote the dissociation of the Atg machinery from complete autophagosomes\textsuperscript{63}. Similarly, Wu and colleagues showed that myotubularin MTM-3 is a positive regulator of \textit{C. elegans} autophagy, promoting autophagosome maturation\textsuperscript{64}. The difference in the role of MTMR phosphatases in mammals and yeast autophagy could reflect a difference between species or simply highlight a possible dual role of phosphatases at different steps of the autophagy process\textsuperscript{64}.

\textbf{1.3.c. The Atg9 recycling system}\n
Atg9 is the only integral membrane protein within the core Atg machinery and possesses six conserved transmembrane domains with the two termini oriented cytosolically\textsuperscript{65–68}. It also presents a conserved motif required for Atg9 self-interaction\textsuperscript{69}. In yeast, Atg9 is recruited at the PAS at early stages of autophagosome formation\textsuperscript{70,71}. Atg9 localizes at the PAS and IM as well as in dispersed cytosolic structures, which are derived from the Golgi and the endosomal system\textsuperscript{72–74}. 
These cytoplasmic pools appear to be clusters of single membrane, highly mobile, Atg9-positive vesicles with a diameter of 30-60 nm and tubules. During starvation they assemble at the PAS to be incorporated into the autophagosomal outer membrane, possibly contributing as one of the membrane sources for the biogenesis and expansion of the phagophore. It is still unclear, however, whether Atg9 cycles to and from the PAS continuously during the formation of an autophagosome or whether Atg9 is only transported to the PAS at its formation and retrieved at the completion of the autophagosome. ATG9A is the mammalian homologue of Atg9. ATG9A-positive membranes interact dynamically with autophagosomal intermediates but they do not become integral part of them, which could indicate that this protein has partial divergent roles in the autophagy within species.

It has been suggested that Atg23 and Atg27, two yeast-specific Atg9 binding partners, function in similar way as Atg11 (see below) in targeting Atg9 peripheral structures to the PAS (Figure 5). Recent work, however, has revealed that they might have a role in the formation of the cytoplasmic Atg9-positive structures as less of these

![Figure 4. PI3K complexes I and II.](image-url)

The two complexes are constituted by 3 common subunits, i.e. Vps34, Atg6 (also known as Vps30) and Vps15, and the Atg14 and Vps38, which are specific for complex I and II, respectively. The PI3K complexes catalyse the formation of PI3P, which recruits its downstream factors that have binding domain for this lipid. Complex I localizes to autophagosomes while Complex II localizes to the endosomes and the vacuole.
structures are observed when \textit{ATG23} or/and \textit{ATG27} are deleted\textsuperscript{71,79}. Atg23 and Atg27 are essential for the Cvt pathway and partially involved in autophagy, and are in complex with Atg9 and they traffic together with this protein\textsuperscript{76–78}. As Atg9 is mislocalized to the vacuolar lumen upon deletion of \textit{ATG27}\textsuperscript{71}, the current idea is that Atg23 and Atg27 promote the appropriate Atg9 sorting from the Golgi, possibly into specialized subdomains, to generate the peripheral Atg9-positive structures\textsuperscript{79}.

Atg11 has been shown to be a downstream effector of yeast homolog of Rab1 Rab GTPase (Ypt1). The interaction of these two proteins is required for the assembly of the PAS during the Cvt pathway\textsuperscript{80}. Interestingly, the Ypt1 GTPase together with its GEF Trs85, a specific subunit of the autophagy-specific transport protein particle III (TRAPPIII) complex, and Atg11, interact on Atg9-containing membranes and at the PAS\textsuperscript{80}. Thus, Ypt1 appears to be specifically activated by TRAPPIII recruiting Atg11 to target the Atg9-containing membranes to the PAS and possibly promote their homo- and/or heterotypic fusion\textsuperscript{80–83}. Wang and colleagues also showed that TRAPPIII binds Atg17 recruiting and activating Ypt1, which in turn participates in the association of Atg1 to Atg9-positive vesicles, and the formed Atg17-Atg31-Atg29/Atg1-Atg13 complex dimer could be a central player in the tethering of Atg9 vesicles with each other or with other membranes\textsuperscript{84}. In this context, it has been shown that under autophagy induction conditions, Atg17 binds Atg9, and mediates its transport to the PAS, in an Atg11 independent manner possibly substituting this latter\textsuperscript{85}. Moreover, the dimerized Atg17-Atg31-Atg29/Atg1-Atg13 pentameric complex binds to Atg9-positive vesicles and mediates their tethering\textsuperscript{86}. Simultaneously, Atg1, through its C-terminal EAT domain, senses and binds to highly curved membranes and through its dimerization, it also tethers vesicles\textsuperscript{18}. Other possible tethering events could be involved in the Atg9 recruitment to the PAS, namely the ones involving the Atg1 complex through the Hop1/Rev7/Mad2 (HORMA) domain in its N terminus of Atg13\textsuperscript{87}.

Atg9 is retrieved from the autophagosomal membranes just before or after the fusion of the autophagosomes with vacuoles\textsuperscript{71,72,88,89}. Although
the specific time point of Atg9 retrieval from the PAS/IM still has to be clarified, some factors that could play a role in this event have been described. The Atg1 and PI3K complexes have been shown that could be involved in the recycling of Atg9 from the PAS/IM to its peripheral pool (Figure 5)\(^68,72\). Moreover, two other core Atg proteins and Atg9 binding partners, i.e. Atg2 and Atg18, appear to also be relevant for Atg9 recycling (Figure 5)\(^72\). In the absence of any of these factors, Atg9 accumulates at the PAS in any growing condition.

Rieter and colleagues showed Atg2 to be recruited to the PAS independently from Atg18, proposing Atg2 to bind firstly to the PAS and Atg18 being subsequently recruited through the interaction with both Atg2 and PtdIns-3-\(\text{P}\)\(^90\). Indeed, Atg2 binds directly Atg9 and its recruitment to the PAS depends on Atg9 and the PtdIns-3-\(\text{P}\) generated by the PI3K complex\(^70,91,92\). Later, Papinski and colleagues revealed that the Atg1 complex role in the trafficking of Atg9 is probably due to Atg18 recruitment mediated through Atg9 phosphorylation by Atg1\(^93\). This phosphorylation, however, is not involved in Atg9 movement, but still, the requirement of Atg1 kinase activity is indicating that Atg1 phosphorylation of other factors may indirectly influence Atg9 trafficking\(^71,94\). Interestingly, a more precise localization of Atg proteins on yeast growing phagophore, has shown that Atg2 and Atg18, as well as Atg9, concentrate at the extremities of this structure and therefore they might interact there\(^95,96\). Therefore, Atg2 and Atg18 may mediate the recycling of Atg9 from these regions of the phagophore, or possibly promoting Atg9-containing membranes to tether in these regions to promote autophagosome formation.

1.3.d. The Atg8 and Atg12 ubiquitin-like conjugation systems

The elongation of the phagophore and the completion/sealing of the autophagosome rely on the function of two ubiquitin-like conjugation systems centered on the ubiquitin-like Atg8 and Atg12. In the first system, yeast Atg12 is covalently conjugated to Atg5 through the activity of Atg7 and Atg10, an E1- and an E2-like enzyme, respectively\(^97\)–\(^99\). The Atg12-Atg5 complex subsequently associates with Atg16 forming a large oligomer that localizes to nascent autophagosomes via Atg16\(^100,101\). The ATG12-ATG5-ATG16L1/Atg5-
Atg12-Atg16 complex is recruited to autophagosomal membranes in mammals through the binding of ATG16L1 to WD-40 repeat protein interacting with phosphoinositides 2 (WIPI2b), one of the Atg18 homologues, and in *Saccharomyces pombe* via the interaction of Atg5 with Atg18\(^{102,103}\).

In yeast, the second ubiquitin-like protein required for autophagosome elongation and closure is Atg8. Atg8 is post-translationally processed by the cysteine protease Atg4, which cleaves its C-terminal arginine exposing a glycine residue (**Figure 6**\(^{97,104}\)). Through an ubiquitination-like reaction mediated by Atg7 and the specific E2-like enzyme Atg3, which also acts as a ligase\(^{105}\), Atg8 is covalently conjugated via its C-terminal glycine to the amino group of phosphatidylethanolamine (PE) present in the growing autophagosomal membranes (**Figure 6**\(^{106,107}\)). This latter step requires the Atg12-Atg5-Atg16 complex, which stimulates Atg3 activity promoting the transfer of Atg8 to its substrate PE (**Figure 6**\(^{108}\)). Upon autophagosome completion, Atg4 reverts Atg8 conjugation by cleaving it from the PE anchor on the autophagosome outer membrane (**Figure 6**\(^{104}\)). This recycled Atg8 can be used again in the formation of new autophagosomes\(^{104}\). Atg8 association with autophagosomal membranes plays an important role in autophagosome expansion, as the amount of Atg8 influences the size of the autophagosome, possibly by mediating tethering and fusion of membranes\(^{104,107,109–112}\). Kaufmann and colleagues showed that Atg12-Atg5-Atg16 complex associated with Atg8-PE can form a scaffold in membranes *in vitro*, a notion that reinforces the importance of Atg8 lipidation in the formation of autophagosomes\(^{113}\).

In mammalian cells, there are several Atg8 homologues, which are divided in two subfamilies: the microtubule-associated protein 1 light chain 3 (LC3) and γ-aminobutyric-acid-type-A-receptor-associated protein (GABARAP)\(^{114,115}\). In human, there are 3 members of the GABARAP subfamily, i.e. GABARAP, GABARAPL1 and GABARAPL2 or Golgi-associated ATPase enhancer of 16 kDa (GATE16); and 3 of the LC3 subfamily, i.e. LC3A, LC3B and LC3C\(^{116–118}\). Kabeya and colleagues were the first to characterize the role of LC3 in mammalian autophagy\(^{110}\). They showed that similarly to yeast Atg8,
LC3, GABARAP-L1 and GATE16 get conjugated to PE and through this mechanism they become associated with autophagosomal membranes\textsuperscript{110}. Additionally, LC3B and GATE16 were also shown to promote tethering and fusion of membranes \textit{in vitro}\textsuperscript{119}. Importantly, Weidberg and colleagues performed RNAi experiments and depleted each member of human ATG8/LC3 family and demonstrated that each one of them regulates a different step of autophagosome formation. That is, LC3 proteins control the elongation step of the IM, whereas GABARAP and GABARAPL2 are implicated in the later steps of autophagosome formation, i.e. sealing or fusion\textsuperscript{119,120}. Another study, however, showed that the conjugation systems are essential for autophagosome closure and for the degradation of the inner autophagosomal membrane, and less important for the elongation and the autophagosome-lysosome fusion\textsuperscript{121}. Also challenging the previous studies, a simultaneous knockout of all the LC3 and GABARAP proteins in HeLa cells did not abolish the formation of autophagosomes, although those were smaller and were forming at a slower rate\textsuperscript{120}.

\subsection*{1.4 The fusion machinery of autophagy}

As previously described, yeast autophagosomes fuse with vacuole while in mammalian autophagosomes fuse first with endosomes, forming the amphisomes, and then fuse with lysosomes, to generate autolysosomes\textsuperscript{122}. Intracellular membrane trafficking requires three main groups of proteins: the Rab GTPases, membrane-tethering complexes and the soluble N-ethylmaleimide sensitive factor attachment protein receptors (SNAREs). Rab GTPases, can recruit different effector proteins, e.g. cargo adaptors to form transport vesicles, motor proteins to move vesicles to their target membrane, and tethering proteins that recruit fusion machinery (SNAREs) to mediate membrane fusion\textsuperscript{123,124}. The presence of different Rab proteins provides identity to membranes. Rab GTPases are activated by GEFs (specific guanine nucleotide exchange factors), which load GTP on specific Rab proteins. Once bound to GTP, Rab GTPases change conformation and bind their effector proteins. Rab GTPases inactivation is achieved by GAPs (specific GTPase-activating
proteins), which stimulate the hydrolysis of the bound GTP into GDP, and release the effector protein from the Rab GTPases\textsuperscript{125}.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{Atg9_cycle.png}
\caption{Representation of Atg9 cycling system. Atg9 is localized to the phagophore and to cytosolic membranous structures derived from the Golgi and the endosomal system. This integral protein is transported to the phagophore through a mechanism involving Atg23, Atg27 and Atg11 and Atg17. In contrast, Atg9 retrieval from the phagophore depends on the Atg1-Atg13 complex, Atg2, Atg18, and the PI3K complex I.}
\end{figure}

Some Rab proteins have been linked to the autophagy process. RAB7 is essential for the late endosome trafficking and fusion with lysosome, is also important for the fusion between autophagosomes and lysosomes\textsuperscript{126,127}. Inhibition of RAB7 recruitment to mature autophagosomes inhibits their fusion with endosomes without affecting endocytosis, highlighting RAB7 role in the autophagosome fusion step with endosomes\textsuperscript{128}. Furthermore the deletion of the guanosine exchange factor complex of Ccz1 and Mon1, which recruits Rab7 to PtdIns-3-P-positive autophagosomes in \textit{Drosophila} fat cells, impairs the autophagosome-lysosome fusion\textsuperscript{129}. RAB33b, a Rab protein localized at the Golgi complex, was also shown to be involved in both the formation of autophagosomes via interaction with ATG16 and the regulation of the fusion step\textsuperscript{130}.

In mammalian cells more than 60 SNARES are responsible for the membrane fusion specificity. Based on their function, SNARES are divided into \textit{v}-SNAREs, which are associated with the donor vesicles,
and t-SNAREs, which are associated with the target compartment. Alternatively, based on their structure, SNAREs are subdivided into Q-SNAREs, which have a glutamine residue as the central amino acid in the SNARE motif, and R-SNAREs, which have an arginine amino acid residue in the same motif. Based on the amino acid sequence of the SNARE domains, Q-SNAREs are further classified into Qa-, Qb- and Qc-SNAREs. These SNAREs form a four-helix bundle (Qa-Qb-Qc and R-SNAREs) that promotes the approaching and fusion of the two adjacent membranes.

Upon autophagosome completion, the Qa-SNARE syntaxin 17 (STX17) is redistributed from cytoplasm onto these vesicles to mediate their fusion of autophagosome with the lysosome. This fusion event requires STX17 binding with its partners Qbc-SNARE SNAP29 and lysosomal R-SNARE VAMP8. In yeast, the Q-SNARES are Vam3, Vti1 and Vam7, and the R-SNARE is Ykt6, and all are required for the autophagosome-vacuole fusion. Furthermore, Vam7 interaction with Atg17-Atg31-Atg29 trimer triggers this fusion process.

Tethering factors bridge membranes and stimulate the formation of SNARE complexes. The tethering complex Homotypic fusion and protein sorting (HOPS), a conserved protein complex, consists of vacuolar protein sorting 11 (Vps11), Vps16, Vps18, Vps33, Vps39, and Vps41. This complex functions as tethering factor to promote autophagosome-lysosome fusion through interaction of some of its subunits with the STX17. RAB7 recruits its effectors such as PLEKHM1 and RILP, which recruit the HOPS complex via interactions with VPS39 and VPS41, respectively, to promote the fusion event. Similar to what happens in mammals, yeast Ypt7, the counter part of RAB7 in this organism, also recruits HOPS but it appears to take place through direct binding to Vp39 and Vps41.

In *C. elegans*, Ectopic P granules protein 5 (EPG5) was found to be a tethering factor and RAB7 effector that determines the specificity of the fusion between the autophagosomes and lysosomes. RAB7 and late endosomal/lysosomal R-SNARE VAMP7/VAMP8 interact and recruit EPG5 to the late endosomes/lysosomes. EPG5 binds simultaneously LGG-1, one of the Atg8 homologs in *C. elegans*, and
the complex STX17-SNAP29 on the autophagosomes. STX17-SNAP29-VAMP8 trans-SNARE complexes is facilitated and stabilized by EPG5, which may in this way promote the autophagosome-lysosome fusion\textsuperscript{137}. Similarly, ATG14L binds the STX17-SNAP29 complex on autophagosomes and promotes the fusion of autophagosomes with lysosomes mediated by STX17-SNAP29-VAMP8, therefore being considered also a tethering factor\textsuperscript{138}. Phosphoinositides appear to also play a role in the autophagosome-lysosome fusion. As previously discussed, PtdIns-3-P clearance from the completed autophagosome by Ymr1 phosphatase is essential for the dissociation of Atg proteins from the autophagosome membrane making the autophagosome competent to fuse with the vacuole\textsuperscript{63}.

Other studies have shown that correct levels of PtdIns-4-P on autophagosomal membranes, and PtdIns-3-P and PtdIns-3,5-biphosphate on lysosomal membranes, play a crucial role in the fusion of autophagosomes with lysosomes\textsuperscript{139–141}.

![Figure 6. Schematic representation of the Atg8 and Atg12 ubiquitin-like conjugation systems.](image)

The Atg8 conjugation system involves the initial cleavage of Atg8 C-terminal arginine by Atg4, to exposing a glycine residue. Atg8 is then activated by the E1 enzyme Atg7 and transferred to the E2 enzyme Atg3, before to be finally conjugated with phosphatidylethanolamine (PE). Atg8 is recycled from membranes by the Atg4 cleavage of the amide bond between Atg8 and PE. The conjugation system of Atg12 involves Atg12 conjugation to Atg5, through the E1 enzyme Atg7 and the E2 enzyme Atg10. The dimer Atg12-Atg5 subsequently interacts with Atg16 forming an oligomer that works as an E3 enzyme for the conjugation Atg8-PE.
1.5. Atg8/LC3 proteins and the autophagy receptors

Atg8/LC3 proteins are conjugated to PE not only at the surface of autophagosomes, but also in the inner lipid bilayer composing these vesicles\textsuperscript{109,112}. This latter Atg8/LC3 pool is responsible for both direct and indirect, i.e. via autophagy receptors, selection of the cargo to be degraded in the vacuole/lysosome lumen during selective types of autophagy. All autophagy receptors recognize features or specific modifications on the targeted intracellular structures, and possess an Atg8/LC3-interacting motif (AIM) or LC3-interacting region (LIR) motif to bind Atg8/LC3 proteins\textsuperscript{142,143}.

The first autophagy receptor to be characterized has been Atg19. This yeast protein binds to Atg8 and the Ape1 complex, targeting this latter to the vacuole during the Cvt pathway\textsuperscript{31,144,145}. Likewise, the Atg32 and Atg30 transmembrane autophagy receptors are required for yeast mitophagy and pexophagy, respectively, as they permit the selective sequestration of mitochondria and peroxisomes by the phagophore\textsuperscript{25,26,146,147}. In mammals, FUNDC1 is a functional counterpart of Atg32 in hypoxia-induced mitophagy\textsuperscript{148}.

p62 is a mammalian autophagy receptor with a broader cargo spectrum. In fact, p62 is able to bind to ubiquitinated proteins, protein complexes, organelles (mitochondria and peroxisomes) and bacteria via its ubiquitin-binding domain. As the rest of the autophagy receptors, it also directly interacts with LC3 and permits to trigger the formation of the autophagosome around the targeted cargo\textsuperscript{149–152}. Additional ubiquitin-binding autophagy receptors, in part redundant with p62, mediate the recruitment of other substrates of autophagic degradation by interacting with Atg8/LC3 proteins. For example, the neighbor of BRCA1 gene 1 (NBR1), similarly to p62, binds different Atg8/LC3 homologues and cargoes such as ubiquitinated mitochondria and aggregates to promote mitophagy and aggrephagy\textsuperscript{153,154}. The nuclear dot protein 52 kDa (NDP52) binds LC3C and ubiquitin coated \textit{Salmonella Typhimurium} to protect cells from and eliminate the bacteria through xenophagy\textsuperscript{155}. OPTINEURIN (OPTN1), in contrast, binds exclusively ubiquitin chains and ATG8/LC3 proteins promoting selective autophagy of ubiquitin-coated \textit{Salmonella enterica}\textsuperscript{156}. Depending on their substrate,
these ubiquitin-binding autophagy receptors can act independently or cooperatively, this latter to probably have a modular system that allow having multiple recognition systems to guarantee the rapid and correct degradation of potentially cell toxic substrates.

2. Regulation of autophagy
Autophagy, similarly to other essential cellular processes, needs to be tightly regulated to ensure that its activity is stimulated at the correct time and towards the precise substrate(s). Numerous studies have helped to understand different mechanisms of autophagy regulation, but this topic is far from being completely understood as a multitude of signals, including those regulating cell fate, differentiation and metabolism, modulate this pathway. Here, I will briefly focus on the currently best characterized signaling pathways controlling autophagy. 

Atg1/ULK1 complex, as previously described (see section 1.3.a), plays a central role in the induction of the autophagosome formation, and phosphorylation of this complex often leads to the inhibition of Atg1 kinase activity and concomitant inhibition of autophagy. Not surprisingly, the Atg1/ULK1 complex is an important regulatory hub of autophagy. TORC1, as previously described, directly phosphorylates Atg13/ATG13 and Atg1/ULK1/ULK2, and in doing so modulates autophagy in response to changes in nutrient availability, growth factors, energy and stress, in all eukaryotes (Figure 7)\(^\text{157,158}\).

In mammal cells, low cellular ATP levels are sensed by the 5’-adenosine monophosphate-activated protein kinase (AMPK), which phosphorylates and activates the activating tuberous sclerosis 2 protein (TSC2), leading to the suppression of TORC1 pathway\(^\text{159}\). In parallel, AMPK phosphorylates ULK1 leading to autophagy induction and as a result, those two modifications lead to an autophagy induction (Figure 7)\(^\text{158}\). Extracellular insulin and insulin-like growth factors, equally, regulate TORC1 through the activation of the PI3K/Akt signaling pathway. Insulin binding to its receptor triggers the recruitment of class I PI3K, which converts PtdIns-4,5-biphosphate into PtdIns-3,4,5-trisphosphate on the inner side of the plasma membrane, leading to the Akt activation by phosphoinositide-dependent protein kinase 1
(PDK1). Activated Akt phosphorylates TCS2 inhibiting the formation of the TCS2-TCS1 complex and increasing the levels of the GTP-bound form of the small GTPase Rheb. Rheb binds then directly and activates TORC1 to negatively regulate autophagy (Figure 7). Ras/PKA signaling is also an essential mechanism to regulate cellular responses to nutrient levels, and its activation is needed to maintain autophagy inactive in growing cells. Upon inhibition of the PKA pathway, Stephan and colleagues observed an induction of the autophagy process in yeast and revealed that PKA and TORC1 both modulate the Atg1 complex activity by phosphorylation of Atg13 at different sites (Figure 7).

Some studies have shown that specific signaling pathways regulating autophagy modulate PI3K activity. Under normal growth conditions, for example, the PI3K complex formation and its kinase activity are inhibited through the interaction of the antiapototic protein BCL2 with BECLIN1, leading to the down-regulation of autophagy. Under starvation conditions, however, Jun N-terminal protein kinase 1 (JNK1), a stress-activated signaling molecule, phosphorylates BCL2 and this modification disrupts its interaction with BECLIN1, leading to autophagy induction (Figure 7).

Multiple signaling molecules such as eIF2α and GCN2, calcium, GTPases and ceramides are also involved in the regulation of autophagy, and some of them are tissue-specific. Some published reviews describe these molecules and their signaling pathways, and provide information about the different mechanisms for autophagy modulation.

3. The physiological roles of autophagy

Autophagy, in yeast, represents mainly a survival mechanism under starvation conditions to supply nutrients and energy to the cell. This is also in part true for high eukaryotes as mice deficient for Atg5 die within 1 day after birth. These animals have reduced plasma and tissues amino acid concentrations and energy depletion, suggesting that provision of amino acids by autophagic degradation is essential for the maintenance of energy homeostasis required to survive.
through early neonatal starvation period\textsuperscript{170}. Similar results were also obtained with \textit{Atg7}-deficient mice\textsuperscript{171}. In higher eukaryotes, however, autophagy carries out numerous other physiological functions, which often are tissue-specific. In this chapter, I will highlight few of them.

\section*{3.1. Autophagy in cell differentiation and development}

During cell differentiation and development, autophagy allows the cell to face a demand for rapid change of its cytoplasmic content and the importance of this function have been unveiled because autophagy-defective organisms exhibit abnormal differentiation and/or development\textsuperscript{172}. In \textit{C. elegans}, for example, BECLIN1 and Atg8 homologues have been shown to be crucial for normal dauer morphogenesis and life-span extension\textsuperscript{173,174}. Furthermore, \textit{BECLIN1} disruption in mice led to early embryonic lethality, reinforcing autophagy implication in early development in mammals\textsuperscript{175,176}. Similarly, silencing of \textit{D. melanogaster} Atg8 homologue DrAut1 disenabled autophagy induction in the fat cells before the pupariation, and is associated with lethality during metamorphosis\textsuperscript{177}.

During erythrocytes differentiation, the precursor cells, the reticulocytes, undergo organelle degradation, and a couple of studies have demonstrated that elimination of mitochondria partially depends on autophagy\textsuperscript{178,179}. On this line, \textit{ulk1}\textsuperscript{−/−} mice display both reduced clearance of mitochondria and RNA-bound ribosomes and increase levels of reticulocytes during erythrocyte differentiation. Similarly, the \textit{atg7}\textsuperscript{−/−} erythrocytes present an accumulation of damaged mitochondria leading to mitochondria loss and severe anemia\textsuperscript{181}. 
Autophagy has also been shown to participate in lymphocyte differentiation. Haematopoietic cell-specific \( atg7^{-/-} \) knockout mice have a significant low levels of B and T cells in the peripheral blood, with an accumulation of mitochondria in T cells, while T cell-specific \( atg5^{-/-} \) and \( atg7^{-/-} \) knockout mice have lower number of peripheral T cells, which also display an accumulation of mitochondria and higher apoptosis\(^{181-183} \).

Figure 7. Schematic representation of the some of the main signaling cascades regulating autophagy. Autophagy can be induced by deprivation of nutrients, hormones and/or energy, and by other signaling cascades as JNK1 integrating other cues. In response to low ATP levels, AMPK stimulates autophagy by activating ULK1 directly or through phosphorylation of TCS2. Nutrient-rich conditions are sensed by Ras/PKA signalling, which leads to an inhibitory phosphorylation of Atg13. The PI3K/Akt signalling cascade negatively regulates autophagy in response to insulin and growth factors. Upon starvation, JNK1 phosphorylates BCL2 disrupting its interaction with BECLIN1, which triggers the induction of autophagy.
During adipogenesis, autophagy contributes to intracellular remodelling of preadipocytes. Absence of Atg5 in MEFs delayed or suppressed adipocytes differentiation, while knockdown of ATG7 or ATG5 in 3T3-L1 preadipocytes inhibited the lipid droplet formation while also decreasing the levels of adipocyte differentiation factors\textsuperscript{184,185}. Likewise, LC3 silencing in different mammalian cell lines impaired the formation of lipid droplets\textsuperscript{186}. Finally, \textit{atg7}\textsuperscript{-/-} knockout mice present smaller adipocytes that accumulate mitochondria\textsuperscript{185}.

3.2. Autophagy as an anti-aging mechanism

Autophagy link with life-span extension has been principally evidenced in studies with \textit{C. elegans}. Inhibition of insulin pathway and dietary restriction increase autophagic activity and simultaneously extends adult lifespan in \textit{C. elegans}, while \textit{ATG} genes are essential for this extension process\textsuperscript{173,187–189}. Furthermore, caloric restriction was demonstrated to reduce the aging effects also in different organisms including in aged humans with improved verbal memory\textsuperscript{190,191}. \textit{Daf-2}, an insulin-like tyrosine kinase receptor mutant, has a significant life-span extension in comparison to the wild type worm. Interestingly, silencing of BECLIN1 (Bec-1) in daf-2 mutants reduced this extension\textsuperscript{192}. Loss-of-function mutations of Atg1 (Unc-51), Atg7, Atg18 and Bec-1, shortened the nematode life span\textsuperscript{173,187}. These mutants also revealed aging signs as well as decline of cellular integrity of the muscle, tissue deterioration, locomotion defects and enlargement of germ cells earlier than in wild type animals\textsuperscript{187}. Inhibition of TORC1 signalling, which lead to a stimulation of autophagy, also prolonged lifespan in different organisms from yeast to nematodes, flies and mice\textsuperscript{193}.

Simultaneously, autophagy inhibition or impairment, triggers the appearance of age associated phenomena such as accumulation of ubiquitinated proteins, peroxisomes, damaged mitochondria, ER stress and the onset of pathologies (neurodegeneration, hepatic carcinoma, adenocarcinomas, lymphomas) in different organisms\textsuperscript{190}. This also underlines that aging is associated with a defect cell quality control\textsuperscript{190}.
The expression of Atg proteins has been reported to decrease in aged tissues, which leads to the concomitant reduction of autophagy flux. For example ATG5, ATG7 and BECLIN1 are downregulated in ageing human brains, and ULK1, BECLIN1 and LC3 levels are down during osteoarthritis\(^{190}\).

### 3.3. Autophagy and cell growth control

Autophagy, under the control of TORC1 signaling, regulates cell growth in response to different signals like amino acid levels, growth factors and energy levels, to guarantee cell growth is adequate in each environmental conditions\(^ {194}\). Under favorable energy and nutrient conditions, TORC1 signaling induces cell growth, partially by suppressing autophagy, but also through the activation of its critical downstream effectors that stimulate ribosome production and protein translation\(^ {195}\). On the other hand, during suboptimal conditions (e.g. cell stress, restraint of nutrients, lower energy status), cell growth needs to be arrested or slowed down to promote cell survival\(^ {194}\). Under amino acid deprivation, TORC1 activity is inhibited, which results in autophagy induction (see section 2)\(^ {194}\). During cellular low energy status, i.e. low ATP levels, the AMPK pathway is activated, which in turn inactivates TORC1 and consequently induces autophagy (see section 2)\(^ {194}\). Autophagy, by turning over cell components, will produce metabolites that will be consumed to generate and reestablish cell energy levels. The importance of autophagy role in cell growth control is also particularly evidenced as dysregulations of this pathway is linked to tumorigenesis. Diverse studies pointed to BECLIN1 being a tumor suppressor gene as the loss of a single allele is found in 40-75% of human prostate, breast and ovarian cancers\(^ {196-198}\). The absence of BECLIN1, in particular, causes DNA damage and genomic instability leading to increased tumor susceptibility\(^ {199,200}\). In support to this notion, it has been shown that BECLIN1 is capable to inhibit tumorigenesis in breast carcinoma and CaSki cervical cancer cells by regulating growth factor receptor signaling\(^ {196,201,202}\). Other autophagy proteins such as ATG4C\(^ {203}\), Bax-interacting factor-1 (Bif1)\(^ {204}\), ultraviolet radiation
resistance associated gene (UVRAG)\textsuperscript{205}, ATG5 and ATG7\textsuperscript{206} have also been shown to act as tumor suppressors. It remains to be clarified the mechanisms through which autophagy constraints cell growth. It is thought that autophagy bulk degradation is the main mechanism to reduce cellular mass, but evidences indicate that its selective degradation may play a role in this, at least partially. In mammals, p62 was shown to participate in TORC1 activation, something that is abolished in p62-depleted cells and causing a decrease in cell growth\textsuperscript{207}. In yeast, autophagy rapidly and selectively degrades ribosomes, which are required for cell growth, to promote cell survival during starvation\textsuperscript{208}.

3.4. Autophagy and immunity
A multitude of studies have shown that autophagy is one of the most ancient innate immune cell responses against pathogens, including bacteria, protozoan and viruses. The growth of a subset of Salmonella typhimurium is favoured in the cytosol of atg5\textsuperscript{-/-} cells\textsuperscript{209}. In neurons, autophagy provides a defence against Sindbis virus infection; in fact Atg5-knockdown leads to delay of viral protein clearance and increased neuron cell death upon the virus infection\textsuperscript{210}. Several autophagy receptors such as p62/SQSTM1, NDP52, NBR1 and OPTN are implicated in the process of targeting pathogens to the autophagosomes by recognizing ubiquitinated proteins at their surface. p62, for example, was shown to participate in the degradation of Salmonella typhimurium, L. monocytogenes, Shigella flexineri, and the Sindbis virus. S. typhimurium autophagic degradation is also mediated by NDP52 and OPTN\textsuperscript{211}.

Pattern recognition receptor (PRRs), such as Toll-like receptors (TLRs), are activated by pathogen-associated molecular patterns (PAMPs) and cellular stress signals called danger-associated molecular patterns (DAMPs), allowing recognition of specific pathogens or stress conditions associated with infection\textsuperscript{212}. Autophagy is involved in the so-called topological inversion mechanism or cross-presentation, where it engulfs and delivers cytosolic PAMPs to endosomal PRRs and to major histocompatibility complex class II (MHC II) compartments for
antigen recognition\textsuperscript{212}. This function of autophagy is important for PRRs activation, T helper activation and for the presentation of self-derived antigens by MHC II complexes in the thyme. In line with this, thymus-specific \textit{atg}5\textsuperscript{−/−} deficient mice display autoreactive T cell infiltration, inflammation in multiple organs and autoimmune colitis, underlying the importance of autophagy in the MHC II antigen presentation\textsuperscript{213,214}.

While the Th1 cytokine interferon-γ (IFN-γ) induces autophagy, interleukin-4 (IL-4) and interleukin-13 (IL-13) produced by Th2 helper cells and thus also defined as Th2 cytokines, antagonize the autophagic control of intracellular \textit{M. tuberculosis}\textsuperscript{215}. This demonstrates that autophagy is an effector of Th1/Th2 polarization\textsuperscript{212}. PRRs recognize PAMPs and induce various pro-inflammatory cytokines such as interleukin-1β (IL-1β) and interleukin 18 (IL-18). Pro-inflammatory response is suppressed by autophagy, as \textit{atg}16\textit{L}1\textsuperscript{−/−} and \textit{atg}7\textsuperscript{−/−} knockout macrophages show enhanced secretion of IL-1β and IL-18 due to accumulation of damaged mitochondria and release of mitochondrial DNA into the cytosol\textsuperscript{211}. In contrast, starvation-induced autophagy promotes IL-1β secretion. Thus, autophagy suppresses pro-inflammatory signalling during basal conditions, but it upregulates this signalling during stress conditions\textsuperscript{211}.

3.5 Autophagy and pathologies

The accumulation of damaged and/or dysfunctional cell structures can be cytotoxic, therefore the role of autophagy housekeeping/quality control is crucial to prevent the development of different human disorders\textsuperscript{216}. In fact, defects in the autophagy have been associated with multiple diseases, including cancer, neurodegenerative, heart and liver disorders\textsuperscript{217}. In this chapter, I will exclusively focus on neurodegeneration, cardiomyopathies and liver diseases to provide an example of the beneficial role of autophagy in preventing severe pathologies.

BECLIN1 levels have been found to be lower in Alzheimer’s disease patients and reduction of this protein leads to accumulation of β-amyloid and neurodegeneration in mice\textsuperscript{218}. Together with the ubiquitin-
proteasome system, autophagy has also been shown to be important for clearance of α-synuclein, which accumulates in Lewy body inclusions in neurons, of Parkinson’s disease patients\textsuperscript{219–223}. Huntingtons’s disease is characterized by the accumulation of mutated huntingtin, which forms inclusion bodies in brain tissues. It has been revealed that autophagy participates in the degradation of these aggregates\textsuperscript{224,225}. In 2004, Ravikumar and colleagues showed that the aggregates formed by mutated huntingtin can sequester mTOR, inducing autophagy to degrade them and reducing their toxicity\textsuperscript{226}.

Few studies have also revealed that autophagy has an important protective role against cardiomyopathies. Depletion of Atg5 or Atg7 in mice led to the development of cardiac hypertrophy with accumulation of abnormal mitochondria and sarcomere structures in cardiomyocytes\textsuperscript{227,228}. During ischemia/reperfusion (I/R) injury, cardiomyocytes undergo programmed cell death, and autophagosome formation and lysosomal degradation is impaired\textsuperscript{229}. Autophagy enhancement by BECLIN1 overexpression in cardiac HL-1 cells (model for I/R injury to the heart), was able to reduce apoptosis activation, highlighting the possibility that autophagy is an important defence against I/R injury\textsuperscript{229}. The protective role of autophagy in cardiomyocytes has also been demonstrated with lysosome-associated membrane protein 2\textsuperscript{-/-} (lamp2\textsuperscript{-/-})-deficient mice, which exhibit severe accumulation of autophagic vacuoles and impaired autophagic degradation, which lead to cardiomyopathy\textsuperscript{230,231}. Altogether, it appears that constitutive autophagy in cardiomyocytes is crucial for protein quality control and maintenance of cellular structure and function, as the accumulation of abnormal structures, especially mitochondria, is linked to dysfunction.

Livers from atg7\textsuperscript{-/-} knockout mice display an accumulation of ubiquitinated protein aggregates, deformed mitochondria and the presence of aberrant structures in the hepatocytes\textsuperscript{216}. Hepatocytes from those animals also shown an impairment in peroxisome clearance\textsuperscript{232}. Autophagy role in the hepatocytes quality control has also been shown in benign liver carcinomas formed in Atg5-deleted mice and in liver-specific atg7\textsuperscript{-/-} knockout\textsuperscript{233}. Mallory-Denk bodies (MDB), which are
composed by abnormally phosphorylated, ubiquitinated, and cross-linked keratins and non-keratin components, are found in hepatocytes of many human liver disorders, as well as in neurodegenerative and muscle diseases. Interestingly, it has been revealed that autophagy participates in the elimination of MDB\textsuperscript{234}. Chronic alcohol consumption leads to an inhibition of autophagy in the liver, suggesting that this pathway is a protective process against liver alcoholic diseases. Autophagosome formation augmented in an acute mouse model of binge alcohol consumption, which was shown to promote damaged mitochondria and lipid droplets removal\textsuperscript{235}. In vitro, autophagy inhibition increases alcohol damage in hepatocytes, while in vivo, the induction of autophagy reduces alcohol-induced lipid accumulation, and autophagy inhibition aggravated steatosis. Autophagy, therefore, protects the liver from alcohol-induced injuries\textsuperscript{211,235}.

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