Chapter 8

General Discussion
Maintenance of homeostasis is one of the biggest challenges for any living cell. Several signalling pathways and processes trigger responses to help the cell to adapt to all adversities that it may face. For instance, cells need to eliminate not only invading pathogens, but also their own content, from single proteins to entire organelles. This is particularly vital when, for example, these components are misfolded or damaged, or when the nutrients become limited and cells have to quickly generate an internal pool of nutrients by recycling their own constituents. A major cell catabolic pathway, autophagy, represents a key process to cope with these stresses. Autophagy can occur in a bulk-manner, when cellular material is randomly targeted for degradation, or selectively, when specific structures are exclusively sequestered by autophagosomes. Autophagy has been extensively studied in yeast, where genetic screens have allowed the identification of conserved proteins involved in the process, the so-called autophagy-related (ATG) proteins. In addition to its amenability to genetic manipulation, another advantage of studying autophagy in yeast is the presence of a selective type of autophagy, the cytosol-to-vacuole targeting (Cvt) pathway. The study of this biosynthetic transport route is a unique tool to acquire insights into the mechanism underlying selective types of autophagy observed in mammals. The biogenesis of autophagosomes is initiated at a specific site, the pre-autophagosomal structure (PAS), where the initial phagophore is formed, elongates and sequesters the cellular material targeted to degradation. The phagophore finally closes and thus generates an autophagosome. The subsequent fusion of autophagosomes with the vacuole leads to the release of the inner autophagosomal vesicles in the vacuole lumen, where it is turned over by hydrolases. This results in the mobilization of metabolites, which are reused as either building blocks or an energy source.

**Chapter 1: General Introduction**

In this chapter I provided a general introduction on the current knowledge about autophagy, from the mechanism and molecular machinery to the general regulation of this process and the roles of autophagy in maintaining homeostasis in cells, organs and entire...
organisms. These latter aspects highlight the relevance of this process in eukaryotes, which is also underlined through the introduction of some pathological conditions caused by an impairment or a defect in autophagy.

Chapter 2: The origin of autophagosomes: The beginning of an end
In the last half century, a lot of conceptual advances in the field of autophagy have been achieved, from the discovery of the ATG proteins and several aspects of the molecular mechanisms responsible for the biogenesis of an autophagosome, to the regulatory pathways controlling autophagy. Despite those advances, there are still multiple questions that are waiting an answer. One of the main challenges in the field is the elucidation of the origin of the lipid bilayers composing autophagosomes. Although cellular organelles have a clear known origin because in most of the cases are emerging from a pre-existing compartment, autophagosomes appear to be formed de novo, making it difficult to distinctly identify their lipid source. In this chapter, we discussed this topic by describing the key studies on the origins of the autophagosomal membranes. The current landscape of experimental evidences points to the participation of multiple organelles, but a consensus is still far from having been reached. Compartments that have a role in providing membranes for autophagosome biogenesis, include the endoplasmic reticulum (ER), the mitochondria, the Golgi apparatus, recycling endosomes and plasma membrane. The fact that there are different possible membrane sources has made this an even more intriguing mystery. This heterogeneity could be explained by different scenarios. One could be that different membrane sources provide the lipids needed in specific steps of the autophagosome formation. Another scenario could be that the origin of the membranes depend on the autophagy-inducing signal\(^2\). There could also be organism or tissue specificities, which have evolved in order to derive lipids from the optimal reservoirs during autophagosome biogenesis. Additionally, the membrane provision could be linked to organelles that are not targeted to degradation during selective types of autophagy.
In conclusion, more studies on this topic are necessary to bring more clarity into this question and ultimately provide a better understanding of the mechanism underlying autophagy.

**Chapter 3: Conserved Atg8 recognition sites mediate Atg4 association with autophagosomal membranes and Atg8 deconjugation**

In this study, we aimed to better understand how Atg4-mediated processing of Atg8 is regulated and consequently how Atg4 modulates autophagy. Atg4 is a cysteine protease that initially cleaves Atg8 to prime it for conjugation with the phosphatidylethanolamine (PE) pool present at the PAS, through the action of the Atg8- and Atg12-conjugation systems. Atg8 recruitment to the PAS is crucial for autophagosome expansion and closure, but also for the specific interaction with autophagy cargo receptors to mediate selective types of autophagy. Atg4 is also responsible for the deconjugation of Atg8-PE necessary for the release of Atg8 from the surface of complete autophagosomes. Because of its crucial functions in autophagy, Atg8 conjugation and deconjugation from PE is a very important hub for the control of autophagosome biogenesis. Atg4 activity at the PAS/autophagosome very likely needs to be tightly regulated, in order to clear Atg8 from autophagosomal membranes at the right moment. However, there are no evidences showing Atg4 recruitment to the forming or complete autophagosomes. In this study, we were able to reveal a transient Atg4 association to the PAS. We also observed that Atg4 significantly accumulates at the PAS when ATG1 was deleted. This result indicated an early association of Atg4 to the PAS or/and an Atg1 role in the regulation of Atg4 recruitment (as negative regulator). To understand which factors influence Atg4 localization to the PAS, we studied Atg4 subcellular distribution in the absence of multiple ATG genes. As only the deletion of components of the Atg8- and Atg12-conjugation systems prevented Atg4 recruitment to the PAS, we concluded that Atg8 is a positive regulator of this event. We then hypothesized that Atg4 direct binding to Atg8 could mediate this recruitment. Indeed, we found that Atg4 interacts with Atg8 in vivo and
we examined the existence of putative LC3-Interacting Region (LIR) motifs in Atg4. We identified four putative LIR sequences and we generated mutant variants for all of them before testing whether they were essential for the interaction between Atg4 and Atg8 *in vivo*. Three of them, i.e. LIR1, LIR2 and LIR4, are involved in Atg4 interactions with Atg8, and therefore we initially considered them as putative LIR motifs (pLIR). However, only one of them, i.e. pLIR2, plays a role in both the Cvt pathway and autophagy. Accurate analysis of the Atg4\textsuperscript{pLIR2} mutant revealed a slight defect in Atg8 priming, but a strong block in Atg8-PE deconjugation, which indirectly causes impairment on the autophagosome biogenesis because Atg8 fails to be recycled.

We also observed that only the Atg4\textsuperscript{pLIR1} and Atg4\textsuperscript{pLIR4} displayed a slight binding defect to primed Atg8. At the same time, when testing *in vitro* the Atg4-Atg8 interaction, Atg4\textsuperscript{pLIR4} mutation but not Atg4\textsuperscript{pLIR2}, highly decreased the association of Atg4 to non-lipidated Atg8. This strongly suggests that pLIR2 is not a LIR motif but a domain that specifically recognizes Atg8-PE. Given that, we named this sequence Atg8-PE Association Region (APEAR). Interestingly, pLIR4 is a *bona fide* LIR motif that binds Atg8 constitutively, similarly to its counterpart in mammalian ATG4B\textsuperscript{7}. We called it C-terminal LIR (cLIR).

In the second part of this study, we investigated whether these two motifs have a cumulative action on Atg4 function by combining the corresponding mutant in a single Atg4 variant. The double mutant Atg4\textsuperscript{APEAR,cLIR} displayed a stronger impairment in Atg4 interaction with Atg8 and a more evident defect in the Cvt pathway compared to the single mutants. Combining these two mutations also enhanced the defect in Atg8 priming and Atg8-PE deconjugation. However, while Atg4\textsuperscript{APEAR} and Atg4\textsuperscript{APEAR,cLIR} led to a complete block in the Atg8 release from its PE anchor *in vitro*, the Atg4 and Atg4\textsuperscript{cLIR} displayed a normal deconjugation kinetic. Altogether, these data demonstrate a cooperation between the APEAR and cLIR binding sites.

Based on these findings we suggest a model in which upon a constitutive post-translational C-terminal cleavage, Atg8 is recruited to the PAS where it is conjugated to its lipid anchor. At the PAS, Atg8 is protected from Atg4 catalytic activity to promote Atg8-PE role in the
autophagosome expansion. Other regulatory mechanisms, however, are not mutually exclusive. Upon autophagosome completion, the release of other autophagy machinery from the outer surface of the autophagosome could make Atg4-recognition sites in Atg8 accessible to interact with cLIR, and with APEAR, which would allow Atg4 to recognize Atg8-PE C-terminal and consequently cleave Atg8. Altogether, our study showed for the first time that Atg4 is recruited to the PAS and Atg4 cleavage of Atg8 and Atg8-PE involves different domains in the protein.

Chapter 4: Atg4 proteolytic activity can be inhibited by Atg1 phosphorylation

In this chapter we further investigated the modulation of Atg4 activity to better understand autophagy regulation. We revealed that Atg4 is phosphorylated by Atg1 \textit{in vivo} and \textit{in vitro} on its serine 307 (S307). The role of this Atg4 phosphorylation was then studied with non-phosphorylatable (S307A) and phosphomimicking (S307D) mutants. Mimicking Atg4 phosphorylation led to the block of both the Cvt pathway and autophagy, while the Atg4\textsuperscript{S307A} mutant did not showed a major defect in these two pathways. We also found that Atg4\textsuperscript{S307D} mutant is proteolytically inactive, i.e. both cleavages are inhibited, while Atg4\textsuperscript{S307A} behaved almost like wild type Atg4. The strong defect in Atg8-PE deconjugation of the Atg4\textsuperscript{S307D} mutant was also confirmed \textit{in vitro}.

Next, we explored how Atg1-mediated Atg4 phosphorylation may regulate Atg8 and Atg8-PE cleavages. We therefore tested whether our Atg4 mutants interfere with the binding between Atg4 and Atg8. Our results demonstrated that while this interaction increases in cells expressing Atg4\textsuperscript{S307A} or a protease-dead version of Atg4, it is disrupted in a strain carrying Atg4\textsuperscript{S307D}. As phosphorylation of Atg4 at Ser307 by Atg1 can inhibit both Atg8 post-translational cleavage and Atg8-PE deconjugation, we then hypothesized that Atg4 phosphorylation by Atg1 occurs at the PAS to modulate Atg8-PE cleavage during autophagosome formation. We detected Atg4 interaction with Atg1 principally at the PAS, which indicates that a regulation of Atg4
cleavage probably occurs at this location. We thus proposed that Atg1 phosphorylates Atg4 at the PAS impeding this protease to interact with Atg8-PE during autophagosome formation. Upon autophagosome completion, Atg1 gets inactivated and/or released from the autophagosome, an event that allows Atg4 to bind Atg8-PE and deconjugate Atg8-PE. However, the mechanism by which Atg1 phosphorylation influences Atg4 binding to Atg8 requires further investigation.

Together chapters 3 and 4, are two important and complementary studies highlighting some of the aspects of the Atg4 regulation at the PAS. We revealed a transient Atg4 recruitment to this location, where Atg4 binds to Atg8-PE and deconjugates it. Atg4-Atg8 interaction on autophagosomal membranes principally occurs through the conserved APEAR motif, which allows Atg4 to recognize Atg8-PE. This interaction is additionally regulated through the cLIR motif. Atg1, however, phosphorylates Atg4 at the Ser307 blocking this interaction and consequently inhibits the release of Atg8 from autophagosomal membranes. The regulation of Atg4 binding to Atg8 and its phosphorylation by Atg1 are both crucial to maintain Atg8-PE at the PAS surface to promote a normal autophagosome expansion. However, we still need to unveil how the phosphoregulation is modulated. Is Atg1 presence at the PAS enough to modify Atg4, or are there other upstream factors responsible for this regulation? Moreover, it remains to be understood the mechanistic connection between this regulation and the APEAR-dependent Atg4-Atg8-PE binding. These could be simultaneous, sequential or mutually exclusive.

Chapter 5: Screening for components of the Atg machinery involved in Atg9 trafficking
Selected components of the Atg machinery have been shown to participate in the trafficking of Atg9 vesicles to and from the PAS. The potential contribution of the rest of the core Atg proteins, however, remains unknown. With the aim to identify which other Atg proteins are involved in the Atg9 trafficking, we performed a screen in yeast making use of gene knockout strains and live-cell microscopy. We were
able to evaluate the participation of each one of the core Atg proteins in both the anterograde and retrograde Atg9 trafficking during Cvt pathway and autophagy. With my work, we could draw a more complete scheme of the proteins involved in Atg9 trafficking. During the Cvt pathway, Atg9 transport to the PAS is assisted by Atg11, and Atg9 vesicle retrieval from the PAS requires Atg1, Atg13, Atg2, Atg14, Atg7, Atg29 and Atg31. During autophagy, however, Atg9 trafficking is regulated in part differently. Its anterograde transport depends on Atg11 and on Atg12, while its retrograde movement is mediated by Atg1, Atg13, Atg2, Atg5 and Atg31. We showed for the first time that members of the Atg8- and Atg12-conjugation systems, in particular Atg5, Atg7 and Atg12, are potentially involved in the Atg9 trafficking. Their role, however, is likely independent from their function in conjugating Atg8 onto autophagosomal membranes. We also showed that this is also the case for Atg29 and Atg31, which appear to function outside the context of the Atg1 kinase complex, and Atg14, which acts independent from the PI3K complex I. It has recently been suggested that Atg9 could play a role in the curvature and/or closure of autophagosomes. We propose that the specific involvement of different Atg proteins in the Cvt pathway or autophagy, mediates not only Atg9 trafficking but could also be associated with Atg9 to dictate specific roles of this protein in the modulation of autophagosome biogenesis, e.g. promoting the formation of bigger autophagosomes during bulk autophagy. These results will raise great interest to initiate new studies aimed at unveiling the function of the identified proteins during Atg9 trafficking. Ultimately, this would be an additional step towards our understanding of Atg9 role in autophagy.

Chapter 6: Lipid partitioning at the nuclear envelope controls membrane biogenesis
The crucial role of phospholipids in cell homeostasis is not only restricted to the formation of cellular membranes, but it also concerns cell growth and proliferation. Moreover, triacylglycerols (TAGs), which are mainly stored in lipid droplets (LDs), provide the energy
required for cell survival during nutrient deprivation. The flux of phospholipids and TAGs must therefore be spatially and temporally regulated to ensure proper cell growth and sufficient energy storage when nutrients are scarce. Different studies have shown that dysregulation of phospholipid homeostasis is linked to the development of cancer, metabolic disorders, type 2 diabetes and obesity. The mechanisms underlying the regulation of lipid flux, however, are still poorly understood. TAGs are synthetized from glycerol and fatty acids at the ER membrane, from where the LDs form and expand. Phosphatidate (PA), a key intermediate, is first dephosphorylated by Mg\(^{2+}\)-dependent PA phosphatases (PAPs) into diacylglycerol (DAG), which is subsequently transformed into TAGs through fatty acid acylation by acyltransferases. PA is also a central precursor for the synthesis of phospholipids and as a result, PAPs regulate a key branching step that guides glycerol backbones and fatty acids to the synthesis of either TAGs or phospholipids. Budding yeast expresses only one PAP enzyme, Pah1, which is essential for TAGs synthesis. As Pah1 lacks transmembrane domains, it has first to translocate onto membranes to generate DAG from PA. Pah1 membrane recruitment and activation depends on Pah1 dephosphorylation, which is catalysed by the highly conserved transmembrane Nem1-Spo7 complex.

In this study, we described the mechanisms underlying how growth and nutrient signals during starvation control Pah1 targeting to membranes. To understand whether the Pah1 localization would direct lipid flux towards storage, we initially used a stable Pah1 form and followed its localization under growing conditions until glucose exhaustion, which mimics starvation. We showed that under low glucose conditions, Pah1 transiently translocates from the cytosol to the nuclear membrane domain in contact with the vacuole, known as the nuclear vacuole junction (NVJ). There, this phosphatase accumulates into two nuclear membrane puncta flanking the NVJ in contact with LDs, suggesting that Pah1 is involved in TAGs synthesis and storage. The relocalization of Pah1 to the NVJ is dependent on the activation of the Nem1-Spo7 complex. We revealed that in absence of enzymes essential for the LDs biogenesis, i.e. the steryl acyltransferases ARE1 and ARE2, and the
DAG acyltransferases *DGA1* and *LRO1*, Pah1 was still targeted to the nuclear envelope, showing that its targeting does not depend on LDs formation. Cells lacking these four enzymes, however, presented membrane proliferation, with nuclear and peripheral ER membrane disorganization under glucose depleting conditions. Therefore, membrane proliferation occurs when Pah1 is activated and LDs biogenesis is impaired.

In summary, under low nutrient conditions, Nem1-Spo7 phosphorylates Pah1 triggering its translocation from the cytoplasm to the nuclear membrane flanking the NVJ, where Pah1 channels DAGs into TAGs production and LDs biogenesis. Pah1 regulation through growth signals represents therefore the hub to switch from phospholipid synthesis and membrane biogenesis to lipid storage, a change that is essential for the cell survival during starvation.

**Chapter 7: A neurotoxic glycerophosphocholine impacts PtdIns-4,5-bisphosphate and TORC2 signaling by altering ceramide biosynthesis in yeast**

Lipids are well recognized as signalling molecules that regulate a multitude of physiological responses. Key cellular processes, including cell proliferation, apoptosis, metabolism and migration, are controlled by signalling lipids such as eicosanoids, phosphoinositides, sphingolipids and fatty acids. Numerous extracellular signals regulate the activity of lipid-modifying enzymes such as phospholipases, prostaglandin synthases, 5-lipoxygenases, phosphoinositide 3-kinases, sphingosine kinases and sphingomyelinases. The downstream targets of these enzymes, constitute a complex lipid signalling network with multiple nodes of interaction and cross-regulation. Different lines of research have shown that disruption and imbalance in lipid homeostasis severely alters cellular functions and contributes to the pathogenesis of human diseases. In fact, a number of studies have demonstrated a link between lipid dyshomeostasis and Alzheimer’s disease (AD) progression. Lipidomic analyses on both post mortem brain tissues
and AD mouse models have revealed drastic changes in lipid species of most of the major lipid subclasses\textsuperscript{23}. Along this line, the levels of 1-O-hexadecyl-2-acetyl-sn-glycerophosphocholine or PC(\textit{O-16:0/2:0}), a lipid second messenger, were found elevated in the temporal cortex of AD patients, in TgCRND8 transgenic mice expressing human familial-disease mutant amyloid precursor protein, and in human neurons directly exposed to soluble A\textsubscript{\textbeta42} oligomers\textsuperscript{24}. Intracellular accumulation of PC(\textit{O-16:0/2:0}) in neurons, stimulates TAU hyperphosphorylation and caspase-dependent cell apoptosis independently from the G-protein-coupled PC(\textit{O-16:0/2:0}) receptor, ultimately compromising neuronal viability\textsuperscript{24,25}. This neurotoxic receptor-independent signalling mediated by PC(\textit{O-16:0/2:0}), however, is still not well understood.

Our collaborators previously described phospholipase D (PLD) (Spo14 in yeast), a phosphatidylinositol 4,5-biphosphate (PtdIns(4,5)P\textsubscript{2})-binding protein, to have a buffering effect against PC(\textit{O-16:0/2:0}), both in yeast and in cultured neuronal cells. In particular, they observed the relocalization of PLD to distinct foci adjacent to the plasma membrane upon PC(\textit{O-16:0/2:0}) treatment, suggesting that this neurotoxic lipid may modulate lipid signalling networks\textsuperscript{26}. In this new study, we contributed to the investigation on the precise consequences of elevated levels of this lipid on cell homeostasis.

First, we found that yeast exposure to high levels of PC(\textit{O-16:0/2:0}) triggers the relocalization of the PtdIns(4)P 5-kinase Mss4 and PtdIns(4,5)P\textsubscript{2}, similar to that of PLD, i.e. to distinct membrane-associated structures at the plasma membrane that we called PtdIns(4,5)P\textsubscript{2}-enriched structures (PES). By investigating the ultrastructure of the PES we found that they represent large invaginations of the plasma membrane that do not appear to involve aberrant association of endocytic or exocytic proteins. The redistribution of PtdIns(4,5)P\textsubscript{2} is in part due to an accumulation of long chain bases (LCBs) and ceramides caused by the PC(\textit{O-16:0/2:0}) treatment.

Second, as the target of rapamycin complex 2 (TORC2), localizes to the plasma membrane and responds to changes in sphingolipid
biosynthesis, we hypothesized that it could also be a target of PC(O-16:0/2:0). Slm1, a PtdIns(4,5)P$_2$ binding protein, interacts with and recruits Ypk1 kinase to the plasma membrane where is phosphorylated by TORC2. We showed that PC(O-16:0/2:0) treatment promotes the translocation of Slm1 from eisosomes to PES, however this translocation is not required for the inhibition of TORC2-dependent Ypk1 phosphorylation. Further work is required to characterize the mechanism(s) by which PC(O-16:0/2:0) affects TORC2 signalling. Altogether, these results revealed that PC(O-16:0/2:0) causes an accumulation of LCBs and ceramides, and contribute to both the redistribution of Mss4 and PtdIns(4,5)P$_2$, and the inhibition of TORC2 signalling. Overall, our data provide a mechanistic insight into how the accumulation of Aβ$_{42}$ oligomers in AD neurons could lead to cellular lipid disturbances and to its consequent neurotoxic effects.

**Concluding remarks**

Within this thesis, we unravelled different aspects of the regulation of autophagosome biogenesis. We mainly focused on the molecular mechanisms regulating Atg4 catalytic activity and Atg9 trafficking during autophagosome biogenesis. We discovered two mechanisms that regulate Atg4 activity, which are essential for the normal autophagosome biogenesis. We also found new components of the Atg machinery involved in the Atg9 trafficking to and from the PAS. Although our findings have advanced the understanding of key aspects of the regulation and mechanism of autophagy, they also open new questions that need to be tackled in the future to have a better understanding of the process of autophagosome biogenesis. Autophagy plays a key role in several pathophysiological processes underlying e.g. cardiomyopathies, neurodegenerative diseases, immunological disorders and cancer. Given this medical relevance, an in-depth understanding of the regulation of autophagy may lead to the identification of attractive new targets for therapeutic interventions.
References