Atg9 cycling: the molecular mechanisms of anterograde and retrograde Atg9 trafficking

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Summary

Autophagy is a cellular catabolic pathway and plays an important role in various diseases such as cancer and neurodegeneration. In autophagy, there is the formation of an double-membrane vesicle called the autophagosome. The only currently known protein responsible for membrane delivery to the phagophore assembly site, where the autophagosome is formed, is Atg9. Atg9 cycles between its reservoirs and the PAS, but the mechanisms of this cycling are poorly understood. Here, I discuss what is currently known about the anterograde and retrograde of Atg9 trafficking in yeast and compare it with trafficking in mammalian cells. Finally, I show a few experiments which provide data on Atg18, one of the proteins involved in the retrograde transport of Atg9.

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CHAPTER 1: FUNCTIONS OF AUTOPHAGY

1.1: Introduction

Autophagy is a cellular catabolic pathway commonly described as self-digestion or recycling; literally 'self-eating' [Levine, 2008]. Autophagy plays an important role in maintaining the normal cellular homeostasis. It can both protect the cell as well as mediate cell death. The importance of this process is highlighted by the fact that autophagy is highly conserved among all eukaryotes, from yeast and to mammals [Mizushima, 2010]. Furthermore, a defect in autophagy causes severe diseases such as cancer, neurodegeneration and specific microbial infections [Mizushima, 2008]. Below, I will briefly describe the established physiological functions of autophagy.

1.2: The different types of autophagy

There are three different types of autophagy (Figure 1): Macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). During macroautophagy, large portions of cytosol and/or organelles such as mitochondria are sequestered into double-membrane autophagosomes, which

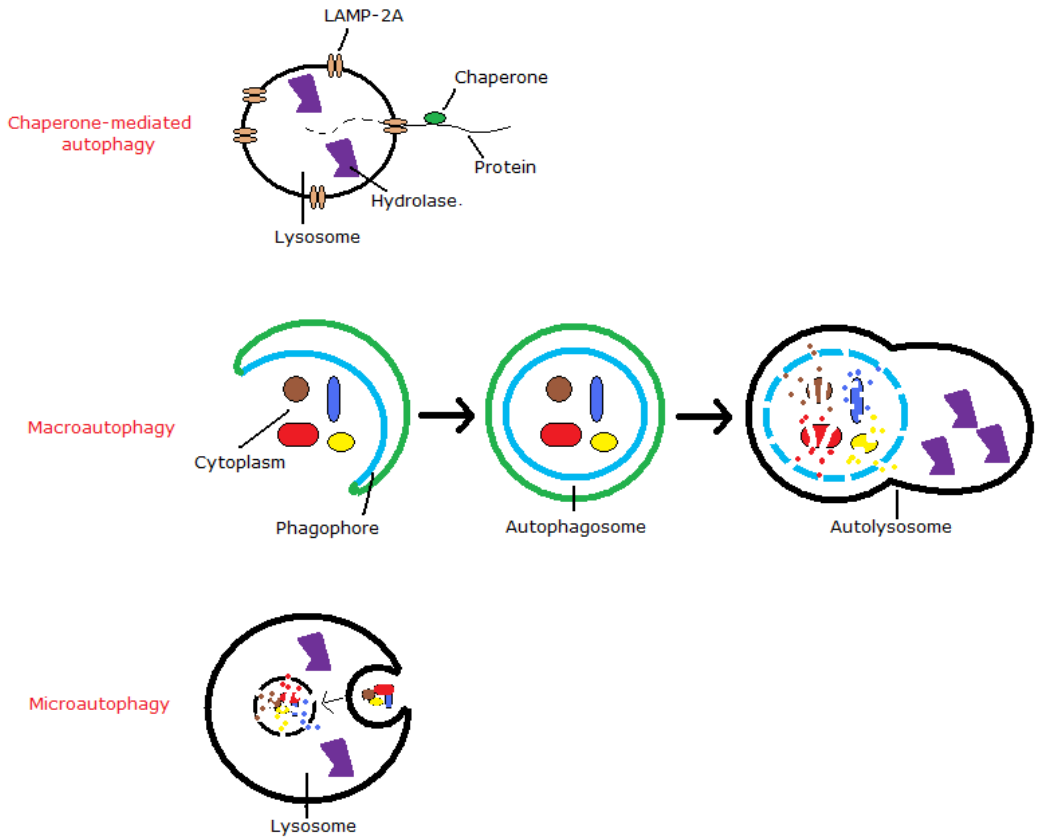

Figure 1. The different types of autophagy. CMA targets specific unfolded proteins with chaperone Hsc70 and transports the proteins across the membrane via the lysosome-associated membrane protein type 2A (LAMP-2A). During macroautophagy, bulk cytoplasm or large unwanted structures are enwrapped into an autophagosome, which is generated from a phagophore. Subsequent fusion of the autophagosome with a lysosome results in the formation of an autolysosome, and the degradation of the inner autophagosome membrane and its content. During microautophagy, the lysosomal membrane engulfs part of the cytoplasm by invagination and pinching off.

then fuse with lysosomes and release their cargo in the interior of this compartment where it is degraded. During microautophagy, lysosomes engulf part of the cytoplasm by direct invagination and pinching of the limiting membrane. During CMA, cytosolic proteins carrying a specific sequence motif are directly imported into the lysosome where they are destroyed [Deretic, 2009].

In this thesis, I will focus exclusively on macroautophagy, hereafter simply called autophagy.

1.3: Cell survival and apoptosis

At first sight, autophagy seems a paradoxical mechanism. On one hand it promotes cell survival in stress conditions, and on the other hand it can induce cell death in some situations. This double-edged sword aspect can be explained by the common factors that these two functions share and therefore they are intertwined and can crosstalk. Little is known about the role of autophagy in both cell survival and cell death. In starvation conditions, autophagy causes cells to lose a large portion of their mass, but they fully recover when shifted back to optimal culture conditions indicating that there is not a so-called autophagy-threshold after which cell death is induced but rather there are some regulatory events that avoid entering this terminal phase [Levine, 2008].

Early observations in embryonic tissues from knockout mice lacking autophagy genes (*ATG)* (Chapter 2) have indicated that apoptosis occurs in more cells relative to the control. Recent studies, however, have demonstrated that this phenomenon should be ascribed to the delayed clearance of cell corpses, since autophagy not only plays a role in cell death, but also in the heterophagic removal of these potentially toxic structures [Deretic, 2009; Qu, 2007]. Overexpression of autophagy genes induces apoptosis, further demonstrating that absence of autophagy does not result in more apoptosis [Mizushima, 2008]. Based on these observations, it seems plausible to conclude that autophagy is normally pro-survival, but when over-expressed or deficient, autophagy may be pro-apoptotic.

1.4: Autophagy and cancer

Cancer is one of the diseases intertwined with autophagy. Oncogenes such as *Bcl2* have been demonstrated to inhibit autophagy, whereas particular tumor suppressor genes such as those encoding for p53 and PTEN stimulate autophagy [Mathew, 2007]. Therefore, it appears that autophagy functions as a mechanism to suppress tumor growth. However, it remains largely unclear how autophagy precisely acts as a tumor suppressor pathway [Mizushima, 2008].

The hypothesis that autophagy suppresses tumor formation is further supported by evidence of the DNA protecting role. It has been demonstrated that the knockout of *ATG5* and *Beclin1* (also known as *ATG6*) results in increased DNA damage in epithelial cells [Levine, 2008]. In this context, an autophagy defect may for example prevent the digestion of ‘leaky’ mitochondria producing reactive oxygen species (ROS) which leads to oxidative stress that, in turn, may cause aggregation of toxic proteins that would normally be cleared via autophagy (or it could cause an energy depletion). These factors contribute to DNA damage. Based on these observations, it is evident that autophagy plays a role in cancer formation and growth.

1.5: Autophagy in immunology

One of the most intriguing systems in which autophagy is involved is the immune system, which is overall divided into two categories: innate and adaptive immunity. Autophagy is part of our innate immunity because it is able to selectively capture and eliminate microorganisms that gain access to the cell cytoplasm in the lysosome. This type of autophagy, termed xenophagy, is able to target bacteria, parasites and viral structures present in the cytoplasm. Autophagosomes formed through xenophagy can be larger than regular autophagosomes to accommodate large or multiple pathogens [Martinez-Vicente, 2007; Mizushima. 2008]. This adaptation indicates a plasticity unique for autophagy that is probably achieved during the formation of autophagosomes (Chapter 1.2). How the autophagic machinery is able to recognize intracellular pathogens remains largely unclear. Recent studies, however, indicate that pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), play a role in the activation of autophagy [Martinez-Vicente, 2007; Mizushima, 2008; Deretic, 2009]. The ROS produced by certain pathogens to induce damage can also activate autophagy and it is likely that this type of activation is similar to the activation of autophagy by ROS in normal physiological conditions [Huang, 2011] or of 'leaky' mitochondria [Dai, 2011].

Recently it has also become evident that autophagy is involved in adaptive immunity as well. Although the precise mechanistic principles remain to be investigated, it has become clear that autophagy contributes to major histocompatibility complex (MHC) class II-antigen presentation of intracellular pathogens by its intrinsic ability of targeting and degrading intracellular molecules, which can subsequently be presented as peptides on MHC class II molecules [Gannage, 2009]. A role for autophagy in MHC class I-antigen presentation has also been demonstrated. In herpes simplex virus type I (HSV-1)-infected macrophages, viral components are targeted by a previously unknown, unique type of autophagy, in which not only double-membrane vesicles are involved, but also four-layered structures [English, 2009]. Both structures are required for antigen processing and presentation on MHC class I molecules to CD8+ T cells, although the mechanisms in this process remain to be investigated [English, 2009]. Similar to MHC class II-antigen processing and presentation, exogenous antigen targeted by the autophagy machinery can be degraded in autolysosomes and the peptides derived from this degraded antigen can be presented on MHC class I molecules. The presentation of processed exogenous antigen on MHC class I molecules is called 'cross-presentation' [Deretic, 2009].

The continuous evolution between pathogens and hosts has lead to adaptations to counter-act each other. This is also the case for autophagy, since it is one of the host defenses against intracellular pathogens. Certain pathogens such as *Mycobacterium tuberculosis* and *Shigella flexneri* have thus developed mechanisms that can effectively hamper degradation through autophagy by inhibiting the biogenesis of autolysosomes [Vergne, 2006], and by avoiding recognition by autophagosomes [Ogawa, 2005], respectively.

1.6: Autophagy in preventing neurodegeneration

Autophagosome accumulation has been demonstrated in several neurodegenerative disorders such as Huntington's, Alzheimer's and Parkinson's diseases [Mizushima, 2008; Levine, 2008] and for long it was thought that autophagy contributed to their pathology. Recent studies, however, have revealed

that autophagy in fact protects neurons against degeneration and that the autophagosome accumulation indicates that autophagy is activated in these cells [Martinez-Vicente, 2007]. These and other studies also hypothesize that in diseases caused by prions, the aberrantly folded proteins that cause damage are targetted by autophagosomes rather than the proteasomes [Nassif, 2011]. The exact mechanisms by which autophagy prevents or reduces neurodegeneration remain unclear.

1.7: Autophagy in ageing and longevity

In ageing cells, there is – among other things – an accumulation of oxidized proteins and damaged organelles. For a long time, it has been postulated that this accumulation is due to an impairment of autophagy. In fact, recent studies have demonstrated that autophagy declines in ageing cells of rodents [Martinez-Vicente, 2007]. Whether autophagy decline is the cause or consequence of ageing still has to be clearly determined. In ageing cells, there are the so-called secondary lysosomes that contain undigested material. Since the fusion of autophagosomes with these lysosomes might result in an accumulation of undigested material, it is not unlikely that these lysosomes are responsible for the decline of autophagic degradation. This might be caused by a competition between the secondary and primary lysosomes in fusing with autophagosomes [Levine, 2008]. The hypothesis that autophagy decline is a cause of ageing is intriguing, since less autophagy probably leads to the aforementioned accumulation of undesired proteins and organelles observed in ageing cells. Nevertheless, whether it is a cause or consequence, autophagy prevents ageing.

Caloric restriction, in other words starvation conditions that trigger autophagy, appears to increase the longevity of organisms. To mimic the effects of caloric restriction, but without the side effects of the starvation conditions, anti-lipolytic drugs are now thoroughly studied in order to develop drugs that efficiently slow down ageing, since it has been demonstrated that these compounds decrease insulin secretion and stimulate autophagy [Donati, 2004].

CHAPTER 2: THE MECHANISMS OF AUTOPHAGY

During autophagy, the target organelles, molecules or bulk cytoplasm are sequestered into a double-membrane vesicle called the autophagosome. When the phagophore fully envelopes the target, it becomes an autophagosome. Complete autophagosomes fuse with lysosomes in mammals or the vacuole in plants and yeast, forming autolysosomes (Figure 1). In the interior of the autolysosome, the internal vesicle of autophagosome and its cargo are degraded by resident hydrolases into basic cellular components such as amino acids, monosaccharides and basic lipids (Figure 1).

2.1: The classification of autophagy

Overall, the autophagy processes are divided into two main categories: Non-selective and selective types of autophagy (see Figure 2).

Non-selective or bulk autophagy, which is often induced by starvation conditions, is characterized by the random sequestration of bulk cytosol and cytoplasmic organelles into autophagosomes. Non-selective autophagy can also be triggered by other cues than starvation conditions, including hormones and growth factors [Nakatogawa, 2009].

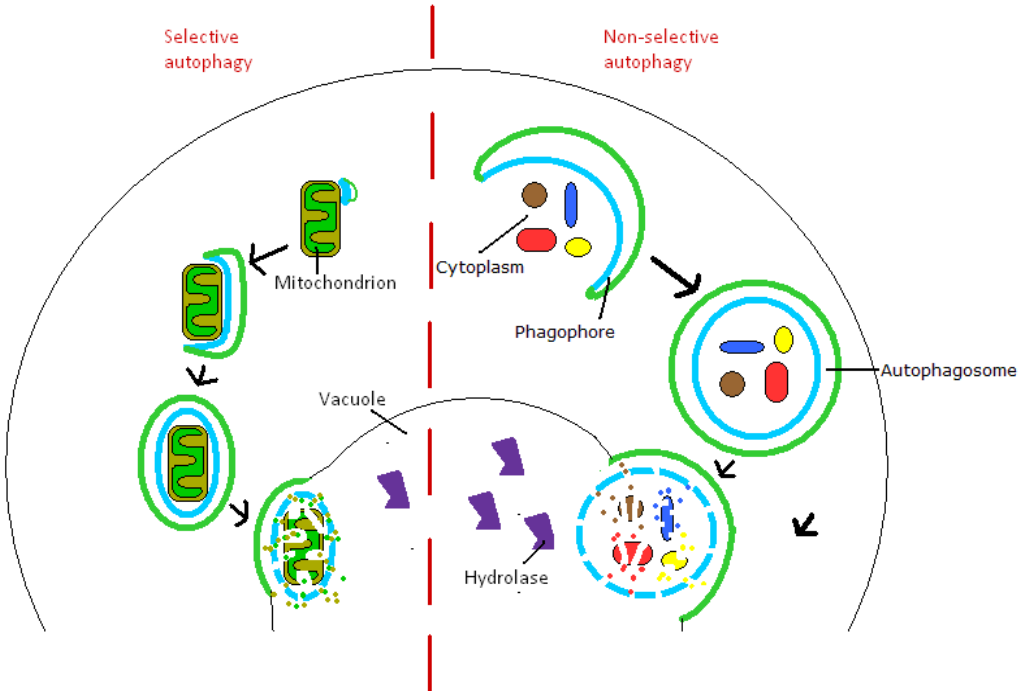

Figure 2. Autophagy classification. Non-selective autophagy (right), encompasses the random sequestration of bulk cytosol into autophagosomes and subsequent digestion in the vacuole-lysosome, after, for example, starvation. Selective autophagy (left) is the sequestration into autophagosomes of specific organelles and/or structures. Mitophagy, the autophagic degradation of mitochondria is pictured here as an example.

Research conducted in yeast on the transport route called the cytosol-to-vesicle (Cvt) pathway has first revealed the existence of selective types of autophagy encompassing the targeting of specific cargo with minimal bulk cytosol into the lysosome/vacuole [Xie, 2007]. In particular, during the Cvt pathway, aminopeptidase 1 (Ape1) forms a large oligomer that is transported from the cytoplasm into the vacuole where Ape1 is proteolitically processed into its active form. The Cvt pathway shares most of its factors with the autophagy pathway and the Ape1 oligomer is also sequestered into a double-membrane vesicle [Xie, 2007]. Recent examples of selective types of autophagy include Atg32-mediated mitophagy, i.e. the autophagic degradation of mitochondria, and Atg30-mediated pexophagy, i.e. the autophagic degradation of peroxisomes [Komatsu, 2010].

2.2: The autophagy (*ATG*) genes

The discovery of autophagy in yeast quickly resulted in the genetic screening for mutants with impaired formation of the starvation-induced autophagosomes, in order to isolate the genes that are involved in autophagy. Currently, more than thirty different *ATG* genes have been identified. It has been shown that the components forming the so-called core machinery, which encompasses 16 *ATG* genes, are essential for all types of autophagy. In addition to this and as mentioned above, the different types of autophagy require particular *ATG* genes. The importance of the core machinery is evident because it is highly conserved in all eukaryoted [Nakatogawa, 2009]. The genes that are considered as part of the core machinery can be divided into four groups: 1) the *Atg1* kinase and its regulators; 2) the class III phosphatidylinositol 3-kinase (PtdIns3K) complex; 3) the *Atg8* and *Atg12* conjugation systems; and 4) *Atg9* and its associated proteins [Yang, 2010].

2.3: The phagophore assembly site (PAS)

The phagophore and subsequent autophagosome formation is proposed to take place at the pre-autophagosomal structure or phagophore assembly site (PAS). Upon induction of double-membrane vesicle biogenesis, the core Atg proteins associate following a hierarchical succession and form the PAS [Yang, 2010; Nakatogawa, 2009]. In absence of the upstream Atg proteins, the recruitment of the downstream Atg proteins at the PAS is blocked [Yang, 2010; Nakatogawa, 2009].

Several of the Atg proteins not belonging to the core machinery but typical of specific selective types of autophagy contribute to the dynamic nature of the PAS. These interactions appear to be crucial in determining the target cargo and therefore guarantee the selectivity of the type of autophagy induced. For example, Atg17, Atg29 and Atg31 are required for starvation-induced autophagy, whereas pexophagy requires a subset of five other Atg proteins [Yang, 2010; Nakatogawa, 2009].

2.4: The Atg1 kinase complex

Atg1 is a serine/threonine kinase, which is thought to play a major role in autophagy type determination. Atg13 is a phosphoprotein and in growing conditions becomes hyperphosphorylated by TORC1 complex [Nakatogawa, 2009]. TORC1 is inactive under starvation conditions or when

treated with rapamycin, and becomes activated after nutrient replenishment [Nakatogawa, 2009]. Hyperphosphorylated Atg13 has low binding affinity for Atg1. Inhibition of TORC1 by starvation conditions leads to a quick dephosphorylation of Atg13, which acquires a high binding affinity for Atg1. Interaction with Atg13 stimulates Atg1 kinase activity, which in turn leads to the induction of autophagy [Yang, 2010; Nakatogawa, 2009; Yang, 2010].

In accordance with its putative role determining the type of autophagy, the Atg1-Atg13 complex interacts with various other proteins, among others with Atg9, Atg17 and Vac8 [Kabeya, 2005]. While Atg9 is essential for all types of autophagy, the latter 2 proteins play a role in autophagy and the Cvt pathway, respectively [Reggiori, 2004].

2.5: The two ubiquitin-like conjugation systems

The Atg8-PE conjugation system generation is as follows (Figure 3). Atg4, a cysteine protease, cleaves the C-terminal arginine residue of Atg8, which allows the E1-like enzyme Atg7 to interact with Atg8 [Xie, 2007;]. Atg8 is then transferred twice; first to the E2-like enzyme Atg3 and finally to phosphatidyl ethanolamine (PE). Atg8-PE oligomerizes and it has been shown that this conjugate can mediate hemifusion of the outer leaflets of membranes [Nakatogawa, 2009]. Atg8 point mutants form smaller autophagosomes compared to the wild type protein, suggesting that Atg8 plays a significant role in the expansion of the phagophore. Interestingly, the transfer of Atg8 from Atg3 to PE is in part catalyzed by the final product of the second conjugation system, the Atg12-Atg5-Atg16 complex [Yang, 2010]. Atg4 is also able to cleave the isopeptide bond between Atg8 and PE, allowing the recycling of Atg8 from membranes [Reggiori, 2005].

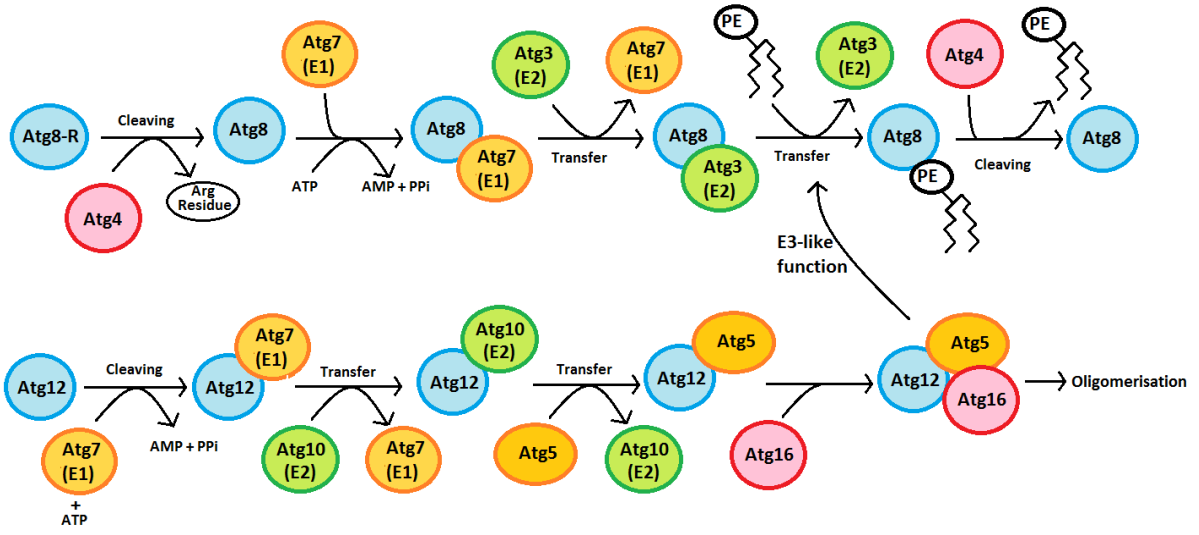

Figure 3. The Atg8 and Atg12 conjugation systems. Atg8 is proteolitically cleaved by Atg4 and it is transferred onto the E1- and E2-like enzymes, e.g. Atg7 and Atg3, respectively, similarly as ubiquitin. PE is transferred to Atg8 through an E3-like activity that requires the Atg12-Atg5-Atg16 complex. Atg8-PE oligomerizes and appears to play a role in the expansion of the phagophores into autophagosomes. Once the autophagosome is completed, Atg4 can cleave the Atg8-PE isopeptide bond permitting the recycling of Atg8. The Atg12 system is similar but not identical to that leading to the formation of Atg8-PE. The E2 kinase is Atg10 instead of Atg3 and Atg5 instead of PE is conjugated to Atg12. Subsequently, Atg16 interacts with the Atg12-Atg5 conjugate.

The Atg12-Atg5 conjugation system is similar to that of Atg8-PE. The E1- and E2-like enzymes are Atg7 and Atg10, respectively, and mediate the transfer of Atg12. Atg12 is finally conjugated to Atg5, forming Atg12-Atg5 complex [Yang, 2010; Nakatogawa, 2009]. This complex associates with Atg16, which, through self-interaction, causes the complex to oligomerize [Xie, 2007]. As mentioned previously, one of the functions of the Atg12-Atg5-Atg16 complex is to participate in the E3-like activity necessary for the formation of Atg8-PE transfer. *In vitro* studies show that only Atg12-Atg5 is required for this function. *In vivo* studies, however, demonstrate that Atg16 is also required, implying that Atg16 does not necessarily contribute to the E3-like function, but is crucial for Atg12-Atg5 localization to the PAS [Nakatogawa, 2009].

2.6: The phosphatidylinositol (PtdIns) 3-kinase complex

The only known class III PtdIns 3-kinase in yeast is the vacuolar protein sorting (Vps) 34. This kinase is able to form 2 different complexes [Kihara, 2001]. Vps34 interacts with Vps15, Vps30 (also known as Atg6) [Xie, 2007] and either Atg14 or Vps38 to form the PtdIns 3-kinase complex I and complex II, respectively [Nakatogawa, 2009]). Complex I is involved in autophagy while complex II plays an important role in the vacuolar protein sorting [Yang, 2010]. Both complexes are able to phosphorylate PtdIns into phosphatidylinositol-3-phosphate (PtdIns3P. Atg14 and Vps38 mediate the complex localization to the PAS or endosomal membranes, respectively [Xie, 2007]. Complex I contributes to autophagsome formation, presumably by recruiting PtdIns3P interacting proteins such as Atg18 [Nakatogawa, 2009]. Atg18 also interacts with Atg2, which is required for Atg9 retrieval [Reggiori, 2005] (Chapter 4).

2.7: The Atg9 complex

Atg9 and its interacting proteins are essential for autophagy. The Atg9 trafficking towards and from the PAS is regulated by various protein complexes. In the next chapter (Chapter 3), I will focus on Atg9 and its putative functions, while in the following ones, the anterograde transport (Chapter 4) and retrograde transport (Chapter 5) will be discussed. Finally, I will discuss what it is known about mammalian Atg9 and compare it with yeast Atg9 data (Chapter 6).

CHAPTER 3: ATG9

Atg9 is the only transmembrane protein that it is part of the core autophagy machinery [Ohashi, 2010; He, 2009; He, 2008; Webber, 2010], and, in contrast to other Atg proteins that are cytoplasmic and transiently associate with the PAS, it localizes to distinct puncta dispersed throughout the cell. The initial evidence was that these peripheral pools of Atg9 were associated with mitochondrial and Golgi membranes [He, 2009; Ohashi, 2010], but recent data suggest that they represent a new organelle (see below). Research shows that Atg9 is able to cycle between these peripheral pools and the PAS [He, 2009]. For the anterograde and retrograde transport of Atg9 to and from the PAS, several different binding partners are employed (Chapters 3 and 4).

3.1: The Atg9 structure

Atg9 consists of 6 transmembrane domains, with the two hydrophilic termini oriented into the cytosol. Its molecular mass is estimated at approximately 130 kDa [He, 2008]. Atg9 was identified in genetic screens designed to isolate mutants deficient for the Cvt pathway and bulk autophagy [Webber, 2010].

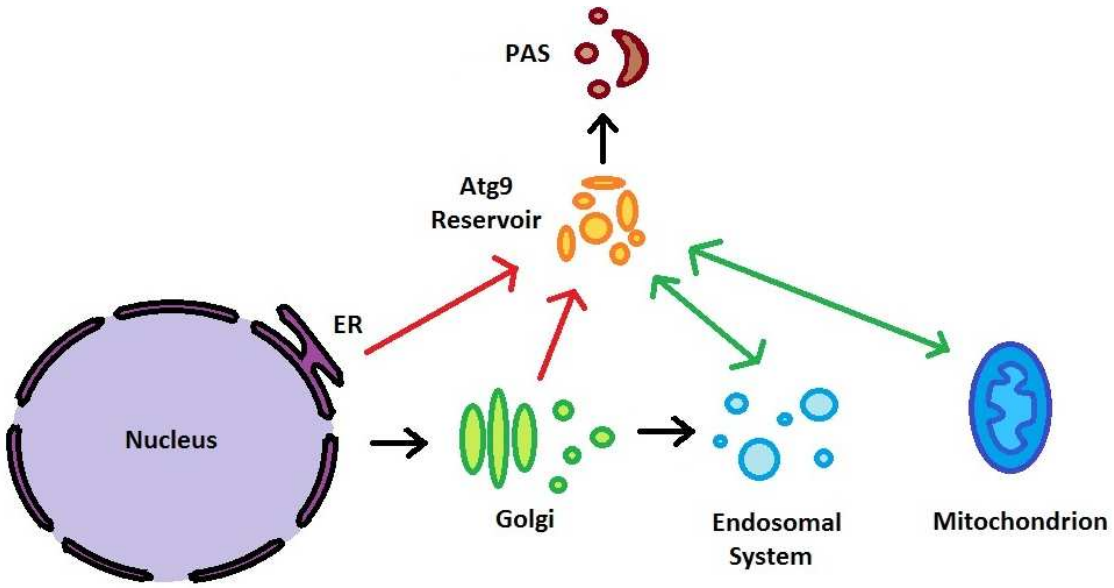

Figure 4. The Atg9 reservoirs. Atg9 exits the secretory system either from the ER, the Golgi, or both (*red arrows*) before localizing to the Atg9 reservoirs. Reservoir components such as lipids can be exchanged with the endosomal system and possibly mitochondria (*green arrows*). These compartments are often found in close proximity of mitochondria but the reason of this association is unknown. The Atg9 reservoir re-localization close to the vacuole leads to the formation of the initial PAS. It remains unclear whether the Atg9 reservoirs also contribute to phagophore expansion.

3.2: The Atg9 reservoirs

Atg9 resides on a few distinct locations in the cell. In yeast, protein markers for the endoplasmic reticulum (ER), mitochondria and the Golgi complex co-localize adjacently with fluorescently-tagged Atg9, especially under starvation conditions, suggesting that the peripheral pool of Atg9 could reside on the membrane of one or more of these compartments [Webber, 2010]. This is partially contradicted by research on mammalian cells, where the mammalian Atg9 peripheral pools are not located on the ER or mitochondria, but only found in the Golgi-endosome system [Ohashi, 2010]. In the case of mitochondria, the proximity may be explained by starvation-induced mitophagy, or by the fact that both Atg9-GFP and mitochondria utilize actin for intracellular positioning [Ohashi, 2010].

Recently, it has become evident that Atg9 may not reside on membranes of known organelles, but instead is concentrated in a new compartment named the 'Atg9 reservoirs' [Mari, 2010]. The Atg9 reservoirs are distinct organelles often found in proximity of mitochondria that are able to exchange at least lipids with the endosomal system [He, 2006]. Atg9 reservoirs originate from the ER and/or the Golgi, but the exact location where Atg9 carriers exit these organelles to be transported to the reservoirs remains an open question (Figure 4). Recent research suggests that at least the Golgi system is involved in Atg9 carrier exit [Ohashi, 2010]. In the Golgi, the conserved oligomeric Golgi complex (COG) might play a role in Atg9 carrier exit, since disruption of this complex results in impaired Atg9 sorting and cycling. It should be noted that the reservoirs and the PAS cannot be easily distinguished so it is possible that the COG only regulates Atg9 trafficking between the Golgi and the PAS and not between the Golgi and the reservoirs [Yen, 2010]. The re-localization of at least one Atg9 reservoir near the vacuole triggers the recruitment of other Atg proteins leading to the formation of the PAS [He, 2009]. Few specific Atg proteins regulate this transport (Chapter 3).

3.3: Atg9 self-assembly

Atg9 has been shown to be concentrated in puncta, suggesting that Atg9 self-interacts with its C-terminal amino acids at positions 766 to 786 [He, 2009]. Research has demonstrated that indeed Atg9 self-interacts and that this association is essential for Atg9 functioning because in its absence, both the Cvt pathway and bulk autophagy are blocked. Atg9 self-association, in particular, is required for its anterograde transport to the PAS and subsequent autophagosome formation (Chapter 3), but not for retrograde transport from the PAS (Chapter 4).

CHAPTER 4: THE ANTEROGRADE TRANSPORT OF ATG9 TO THE PAS

As discussed in the previous chapter, Atg9 is able to self-interact and this self-association promotes Atg9 trafficking from peripheral pools to the PAS [He, 2008]. Using fluorescence microscopy and the Atg9 mutant lacking the 4 conserved amino acids in the C-terminus of Atg9 that are required for Atg9 self-assembly, another function of Atg9 has been discovered. When Atg9 is not able to self-interact, the phagophore formation appears fragmented, e.g. the cells have multiple smaller phagophores. This suggests that Atg9 not only is required for membrane delivery to the PAS, but also for the tethering and subsequent fusion of these membranes to form the phagophore (Figure 5) [He, 2009].

Atg9 recruitment and transport towards the PAS is also tightly regulated by 3 Atg proteins: Atg27, Atg23 and Atg11.

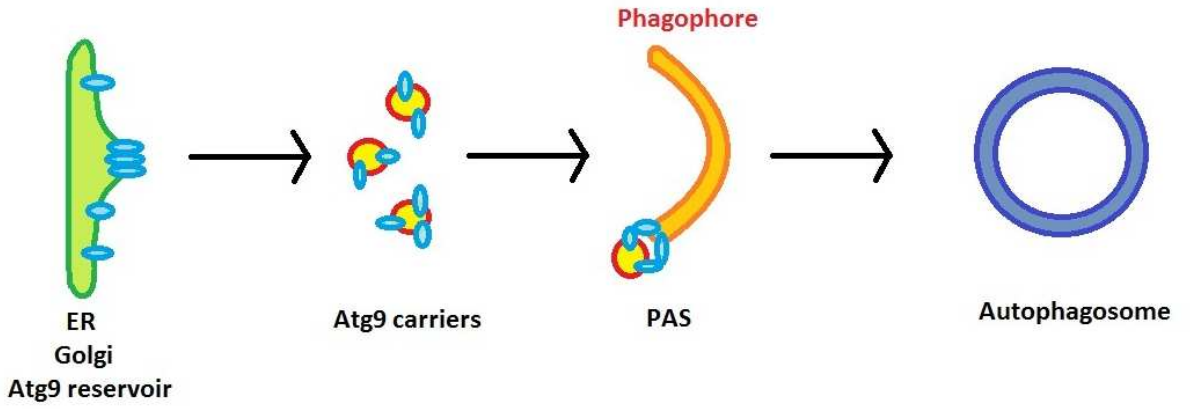

Figure 5. Atg9 functions. Atg9 has at least two distinct functions, e.g. supplying the PAS with membranes and the tethering and/or fusion of those to form the phagophore. Atg9 is mostly concentrated in the Atg9 reservoirs. From this source, only membranes with self-associated Atg9 appear to move towards the PAS where they associate with the phagophore.

4.1: Atg27

Atg27 is an integral transmembrane Atg protein not belonging to the subset of the core Atg proteins. It was originally termed Etf1 and was thought to be a PtdIns3P-binding protein, acting downstream of the PtdIns 3-kinase Vps34 [Wurmser, 2002]. Recent research, however, has demonstrated that Atg27 is a type I integral membrane protein, with the proposed PtdIns3P-binding site in the lumen, which is therefore unable to bind PtdIns3P which is always present on the cytosolic surface of membranes [Yen, 2007]. The N-terminus of the type I transmembrane Atg27 contains the signal peptide, which is cleaved once Atg27 is translocated into the ER [Yen, 2007].

4.2: The Atg27 roles in different types of autophagy

To investigate the role of Atg27 in autophagy, an *atg27Δ* deletion strain was generated and the resulted cells were analyzed [Yen, 2007]. The precursor Ape1 (prApe1) processing into mature Ape1 (mApe1) was defective revealing that Atg27 is essential for the Cvt pathway progression. Examination of the precise step of the Cvt pathway in which Atg27 is involved has revealed that Atg27 functions in the double-membrane vesicle formation/completion, since prApe1 is not fully protected against exogenously added proteases in *atg27Δ* cells.

To test the relevance of Atg27 in pexophagy, Pex14 degradation in *atg27Δ* cells has been investigated [Yen, 2007]. In this strain, there is a delay in pexophagy suggesting that Atg27 contributes to, but is not required, for pexophagy. In *atg27Δ* strains, there is also a partial block of bulk autophagy [Legakis, 2007]. Autophagosomes are not reduced in size, however, they are delayed in formation, suggesting again that Atg27 is not essential for autophagy, but contributes to the rate of vesicle formation.

4.3: The Atg9-Atg27 interaction

Atg27 tagged with a fluorescent protein co-localizes with protein markers for the PAS, mitochondria and the Golgi, but not with those of the ER or peroxisomes [Yen, 2007]. Atg27 cycling occurs in an Atg9 and Atg1-Atg13 complex-dependent manner [Yorimitsu, 2005]. In addition, Atg9 regulation of Atg27 is upstream of Atg1, and Atg9 and Atg27 depend on each other for transport from the reservoirs to the PAS [Yen, 2007]. Atg9, however, is able to partially re-localize to the PAS in absence of Atg27, indicating that Atg9 trafficking does not entirely depend on Atg27.

Atg27 might play a role not only in Atg9 transport, but also in membrane delivery, together or not with Atg9. This notion is supported by the fact that Atg27 is a transmembrane protein and localizes to the Golgi system, which is proposed to contribute to membrane delivery to the PAS [Yen, 2007].

4.4: Atg23 and its role in different types of autophagy

Atg23 is a second Atg protein involved in the anterograde transport of Atg9. Atg23 consists of 453 amino acids, with a molecular mass of approximately 51.5 kDa [Meiling-Wesse, 2004]. Its amino acid sequence suggests the presence of coiled-coil domains, as well as the presence of a putative ATPase domain.

The *atg23Δ* cells have been used to study the role of Atg23 in autophagy. The Cvt pathway is defective in the *atg23Δ* mutant because the prApe1 processing into mApe1 is blocked in growing conditions [Tucker, 2003; Legakis, 2007; Meiling-Wesse, 2004]. However, this impairment of prApe1 processing was restored when cells were starved to induce autophagy. Interestingly, by analyzing the size and number of autophagic bodies by EM, the inner single membrane vesicles of autophagosome resulting from the fusion of these latter with the vacuole, has revealed that the number of these structures is reduced [Tucker, 2003]. This suggests that Atg23 is not essential, but contributes to autophagy.

Pexophagy, in contrast, remains normal in absence of Atg23 [Tucker, 2003]. In conclusion, in *atg23Δ* cells not only the Cvt pathway is blocked, but autophagy is also in part defective.

4.5: Atg23 interactions

Atg23 is able to directly interact with Atg9 [Tucker, 2003; Legakis, 2007]. As mentioned previously, Atg27 is also required for Atg9 trafficking, by interacting with Atg9. It has been proposed that since Atg23 and Atg27 both interact with Atg9, these two proteins might bind with each other. Yeast two-hybrid assays confirmed this hypothesis, although the presence of Atg9 is essential for this interaction [Legakis, 2007]. Therefore it has been concluded that Atg9 interacts with both Atg23 and Atg27, and Atg23 and Atg27 only interact with each other through Atg9 and not directly forming a complex (Figure 6).

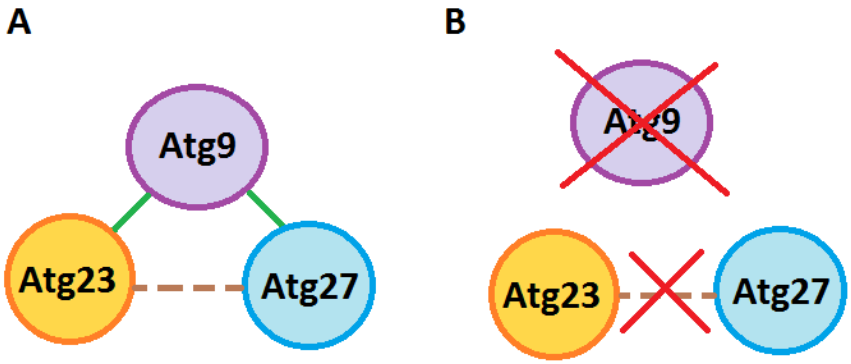

Figure 6. Atg23 interaction. A) Atg9 forms a complex with both with Atg23 and Atg27. Atg23 and Atg27 display a weak interaction (*dashed line*) in between them in presence of Atg9. B) In absence of Atg9, the Atg23-Atg27 interaction is lost indicating that Atg9 is essential for the Atg23-Atg27 interaction.

4.6: Atg23 functions

Atg23 is a peripheral membrane protein and interacts with Atg9. Combined, these arguments indicate that Atg23 might be involved in the supply of membranes for autophagosomes. The *atg23Δ* cells are resistant to inhibition of autophagy by the PtdIns3 kinase inhibitor wortmannin [Meiling-Wesse, 2004], which implies an additional function for Atg23. However, further research is needed to identify this other role of Atg23.

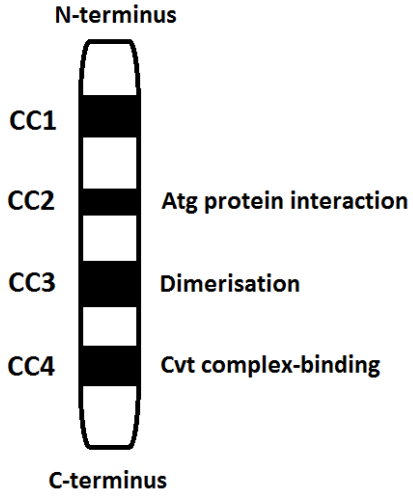

Figure 7. Atg11 structure. Atg11 has 4 coiled-coil (CC) regions, each one with a different function. CC2 interacts with various Atg proteins such as Atg1, Atg17 and Atg9; CC3 is required for the dimerization and CC4 binds the Cvt complex.

4.7: Atg11

Atg11 is a third protein involved in the anterograde transport of Atg9. Atg11 is required for the Cvt pathway, but not bulk autophagy [Reggiori, 2005; He, 2006]. The amino acid sequence of Atg11 shows the presence of 4 coiled-coils (CC) regions. Molecular analysis of these regions revealed different functions (Figure 7): CC2 is able to interact with various Atg proteins, including Atg1, Atg17, Atg20 and Atg9; CC3 enables Atg11 to dimerize and CC4 binds the Cvt complex consisting of prApe1 and Atg19 [Monastyrska, 2006]. Interestingly, it has been hypothesized that Atg11 is similar to Myo2, the yeast myosin V protein, which also has a CC that, like the Atg11 CC3, mediates its dimerization. The C-terminus of Myo2 is functionally similar to the Atg11 CC4, because it is also involved in binding the cargo and the Cvt. In contrast to Myo2, however, Atg11 does not contain an N-terminal motor domain necessary for actin movement, suggesting that Atg11 does not directly bind and move along actin cables [Reggiori, 2005]. Recent research on the actin-related protein 2 (Arp2) shows that this factor co-localizes with Atg9 and it is involved in the anterograde movement of Atg9 [Monastyrska, 2008]. Accordingly, the *arp2* mutant causes an impairment of Atg9 trafficking to the PAS. Further evidence about the Atg9-Arp2 interaction was provided when research proved that that when Atg9 is not able to interact with Atg11, Atg9 does not associate with Arp2. Taken together, these observations indicate that Arp2 links the Cvt pathway with actin, by interacting with Atg9 through Atg11 [Monastyrska, 2008].

4.8: The Atg11 complexes

With its CC domains, Atg11 is able to interact with at least two Atg protein complexes [Yorimitsu, 2005], e.g. the Atg1 (consisting of Atg1, Atg11, Atg13, Atg17, Atg20, Atg24 and Vac8) and the Atg19 complex (consisting of Atg19, prApe1 and Atg11). The Atg1-Atg11 complex is one of the factors able to induce double-membrane vesicle formation, although the Atg1-Atg11 interaction is not essential for autophagy, since yeast *atg11Δ* cells displayed normal autophagy in starvation conditions [He, 2006; He, 2007]. With its CC2 region, it is able to act as a scaffold for Atg1 complex formation, after which the complex is transported towards the PAS (Figure 8) [Yorimitsu, 2005]. The CC4 region catalyzes Atg19 complex formation [Monastyrska, 2006], after which Atg11 re-localizes the complex towards the PAS [Yorimitsu, 2005].

Atg11 also interacts with Atg9 on one hand and the Cvt complex on the other hand. As a result, Atg11 movement toward the PAS assures that during the Cvt pathway, the cargo, e.g. the Cvt complex, and one of the factors regulating the PAS biogenesis, e.g. the Atg9-positive membranes, coordinately reach the site where a double-membrane vesicle will be formed. Yeast *atg23Δ* and *atg27Δ* cells displayed reduced Atg11-Atg9 interaction [Legakis, 2007; He, 2007], demonstrating that Atg23 and Atg27 facilitate this association, although they are not required. The precise interaction sites for the Atg11 and Atg9 association are the CC2 and N-terminal amino acids 159-255, respectively [He, 2006].

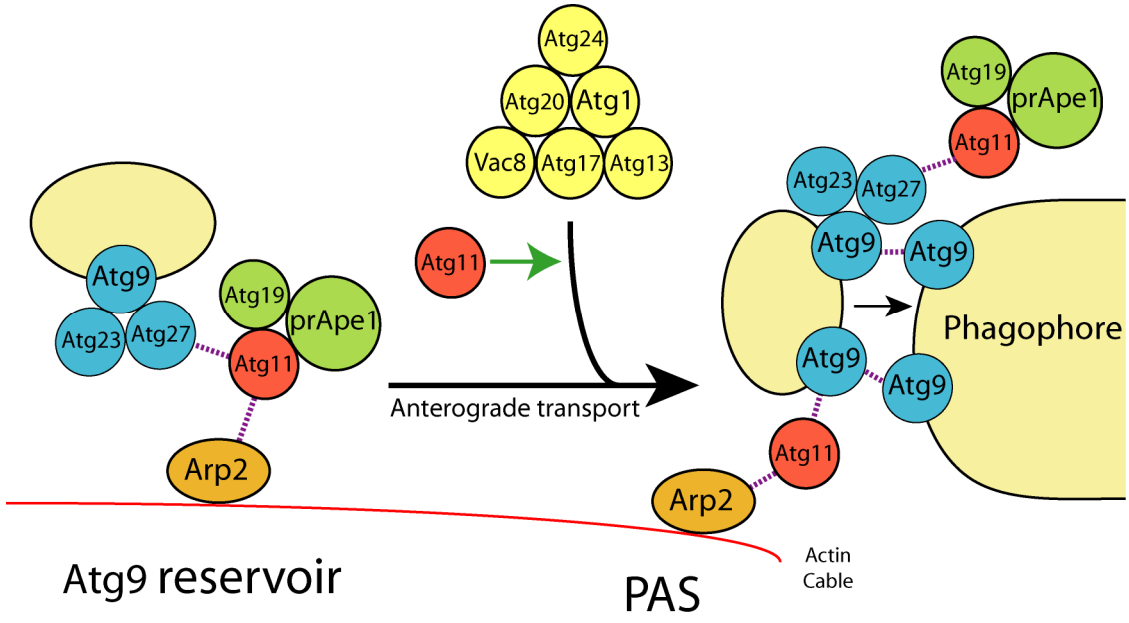

Figure 8. Atg11 functions. Atg11 interacts (purple dashed lines) with the Atg9-Atg23-Atg27 complex (blue) at the reservoir near mitochondria after which the super-complex is transported along actin cables (red) by interacting with Arp2 towards the PAS. Atg11 also recruits (green arrow) the Atg1 complex (yellow) to the PAS to facilitate autophagosome biogenesis, although this interaction is not essential for autophagy. Here, the Cvt pathway is illustrated, where Cvt complex (green) is recruited simultaneously with the Atg9 complex.

4.9: Atg11 localization

In wild type cells, Atg11 localizes to the PAS [He, 2007]. Overexpression of Atg11 confines Atg9 to the PAS, demonstrating that Atg11 overexpression has a dominant effect on Atg9 concentration at this location [He, 2006]. This suggests that Atg11 only facilitates the anterograde transport of Atg9. As mentioned previously, Atg11 is required for the Cvt pathway, but bulk autophagy remains unaffected in *atg11Δ* cells [Reggiori, 2005; He, 2006]. This indicates that Atg11 is not involved in bulk autophagy, perhaps because of its Cvt complex-binding CC3 domain, which makes it highly specific for the Cvt pathway, or Atg11 is redundant with another protein in bulk autophagy and when absent, it could therefore be replaced by other proteins such as Atg17 [Kabeya, 2005]. The former hypothesis is supported by the evidence that yeast cells where actin filament formation is inhibited with a specific drug, have a defect in the Cvt transport route but not bulk autophagy [Reggiori 2005; He, 2006]. In these cells, Atg11 localizes to the reservoirs rather than the PAS [He, 2006]. A model for Atg11 trafficking would be then as follows. During the Cvt pathway, for example, Atg11 binds the Cvt complex and the Atg9-Atg23-Atg27 complex at one of its reservoirs. Atg11 acts as the scaffold in this supercomplex consisting of the Atg9-Atg23-Atg27 complex, the Cvt complex and Atg11 itself. Finally, this entire complex is transported along actin cables near the vacuole forming the PAS, where the Atg1 complex is recruited to generate an active PAS (Figure 8) [Mari, 2010].

CHAPTER 5: THE RETROGRADE TRANSPORT OF ATG9

Atg9 is required for Cvt vesicle and autophagosome formation, but it is absent from the complete vesicle suggesting that Atg9 is retrieved before its fusion of these structures with the vacuole [He, 2009; Reggiori, 2005]. Atg9 self-association is important in anterograde transport (see Chapter 3), but not for the retrograde transport back to the reservoirs [14 He, 2009].

The recycling or retrograde transport of Atg9 from the PAS to the reservoirs is thought to be regulated by 3 protein complexes, e.g. the Atg1-Atg13, the Atg2-Atg18, and the PtdIns 3-kinase complex I (Chapter 1.2). All 3 complexes are required for effective bulk autophagy, as well as for the Cvt pathway, implying that the step of retrograde Atg9 transport does not contribute to determination of the type of autophagy [Kabeya, 2005; Obara, 2008; Obara, 2006]. The Atg1-Atg13 and the PtdIns 3-kinase complexes, however, play a role in an earlier step in autophagosome biogenesis as well, where they regulate the initial step of the double-membrane vesicle formation [Reggiori, 2004; Obara, 2005].

5.1: The Atg1-Atg13 complex

To investigate the role of Atg1-Atg13 on Atg9 trafficking, yeast *atg1Δ* or *atg13Δ* mutant strains expressing either fluorescent-tagged Atg9 or Atg23 were generated [Reggiori, 2004]. As mentioned in Chapter 3, Atg23 co-localizes with Atg9 at both the reservoirs and the PAS, thus being an effective marker protein for Atg9 localization. In the *atg1Δ* mutant, Atg9 and Atg23 were restricted to the PAS. Fluorescent signals in the mutants accumulated over time, indicating that Atg9 and Atg23 were not degraded but indeed remained at the PAS, suggesting that the Atg1-Atg13 complex is essential for Atg9 retrograde transport [Reggiori, 2004].

5.2: The Atg1 kinase activity

The binding partners and/or the kinase activity of Atg1 probably determine the type of autophagy that is performed [Reggiori, 2004; Kabeya, 2005], and in this context, the state of Atg23 phosphorylation [Kabeya, 2005] may contribute to this determination, possibly by guiding Atg1 upon interaction or dissociation. The Atg1 kinase activity is proposed to be crucial in inducing autophagy [Reggiori, 2004], but few works have shown contradictory results by providing data that high kinase activity is essential for either the Cvt pathway and autophagy [Abeliovich, 2003; Kamada, 2000]. However, recent research on a strain carrying a point mutation in a*tg1* that renders the protein incapable of exerting its kinase activity, shows that although Atg23 was confined to the PAS, Atg9 localization was unchanged compared to wild type strains [Kabeya, 2005]. Thus, although Atg1 kinase activity is required for Atg23 localization and possibly Cvt/autophagy determination, there are no (visible) effects on Atg9 retrograde transport.

5.3: The Atg2-Atg18 complex

The second complex responsible for Atg9 retrograde transport consists of Atg2 and Atg18. Atg18 and Atg2 are peripheral membrane-associated proteins and when visualized using fluorescence microscopy, they co-localize with prApe1, which is often used as a marker protein for the PAS [Obara, 2008]. These two proteins also form a complex [Obara, 2008].

5.4: The function of the Atg2-Atg18 complex

This complex interacts with both Atg1 [Tanida, 2011] and Atg9 [Tanida, 2011]. While this complex co-localizes with marker proteins for the PAS in wild type cells, Atg2 and Atg18 can no longer be detected using fluorescence microscopy at this location in *atg1Δ* and *atg9Δ* strains, indicating that Atg1 and Atg9 are essential for Atg2-Atg18 recruitment to the PAS membranes [Obara, 2008].

In yeast cells lacking either Atg2 or Atg18, Atg18 or Atg2, respectively, do not co-localize with marker proteins for the PAS indicating that these two proteins are interdependent for their recruitment to this specialized site [Obara, 2008]. Atg9 was confined to the PAS in both mutant strains, demonstrating that, like the Atg1-Atg13 complex, the Atg2-Atg18 partnership is also essential for the retrograde transport of this protein [Obara, 2008]. It has been demonstrated that Atg18 is able to directly interact with Atg9, only if Atg1 and Atg2 are present, showing that these proteins facilitate this interaction [Stromhaug, 2004]. It is unknown whether Atg2 only strengthens the interaction between Atg9 and Atg18, or whether it represents the subunit of the Atg2-Atg18 complex that directly binds Atg9.

As mentioned above, Atg1 presence is required for Atg2-Atg18 complex recruitment to the PAS. In absence of Atg2, Atg18 is no longer present at the PAS, but it is still present on endosomes [Obara, 2008]. Together with the notion that Atg2 facilitates the Atg9-Atg18 interaction, one could hypothesize that Atg18 is responsible for the correct localization of the complex, while Atg2 mediates the association of Atg9 to Atg18, after which Atg9 is recycled [Tanida 2011]. Experimental evidence, however, suggests a different scenario (see below) because Atg18 and Atg2 recruitment to the PAS is not only dependent on the Atg1 complex, but also on the PtdIns 3-kinase complex I [Xie 2007].

5.5: The PtdIns 3-kinase complex

In absence of Vps38, localization of fluorescent-tagged Atg9 and Atg23 is similar to wild type cells, showing that the PtdIns 3-kinase complex II, which contains Vps38 (Chapter 1.2), is specific for vacuolar protein sorting and not autophagy [Obara, 2008]. In the same manner, cells deficient for Atg6 do not display correct autophagy, indicating that this protein is an essential component of the PtdIns 3-kinase complex. In *atg14Δ* cells, Atg23 localization was similar to wild type cells [Tucker, 2003], but Atg9 was constricted to the PAS, with the exception of small puncta in the cytoplasm [Tucker, 2003]. These puncta can be explained by either the assumption that a small portion of Atg9 at the PAS is able to be recycled back to the reservoirs, or because PtdIns 3-kinase is also required in an earlier step of autophagosome biogenesis by being involved in the anterograde transport of Atg9 [Obara, 2008].

Interestingly, the Atg2-Atg18 complex recruitment to the PAS depends on both the Atg1-Atg13 complex and PtdIns 3-kinase complex I [Obara, 2006] suggesting that these two factors regulate Atg2-Atg18 complex facilitating Atg9 retrograde transport.

CHAPTER 6: MAMMALIAN ATG9 TRAFFICKING

6.1: mAtg9

Most research on autophagy has been performed in yeast, which has been proved to be a suitable model for these studies. Autophagy, however, has additional functions in higher eukaryotes. This chapter focuses on the anterograde and retrograde transport of the mammalian Atg9, mAtg9. Some mammalian homologues/orthologues of yeast proteins regulating the trafficking of Atg9 are listed in Table 1.

Table 1. Mammalian homologues and orthologues of the yeast proteins.

Atg1	ULK
Atg6	Beclin1
Atg8	LC3 / GABARAP / GATE-16
Atg9	mAtg9
Atg13	mAtg13
Atg14	Atg14L / Barkor
Atg17	FIP200
Atg18	WIPI-1
Fab1	PIKfyve
Tlg1p	Syntaxin6
Vps15	p150
Ypt6p	Rab6

The mAtg9 has 2 isoforms, e.g. Atg9L1 and Atg9L2 [Tooze, 2010]. Since the latter is present on placental and pituitary gland tissues only [Tanida 2011], most of the studies have focused on Atg9L1 because it is present in all tissues [Young, 2006]. Hereafter, I will refer to Atg9L1 as mAtg9. This protein localizes to 2 subcellular compartments, e.g. the juxta-nuclear region, or in peripheral pools, where it co-localizes with trans-Golgi Nework (TGN) marker proteins and endosomal marker proteins such as Rab5, Rab7 and Rab9, respectively [Webber, 2007]. Like yeast Atg9, mAtg9 can be found using fluorescence microscopy on forming autophagosomes, which are cup-shaped elongations of the ER termed omegasomes in mammals and could represent the mammalian PAS [Simonsen, 2008]. The structures can be identified, in between others, using the GFP-LC3 construct [Polson, 2010]. In contrast to yeast however, mAtg9 has also been demonstrated to be present on mature autophagosomes, which can be distinguished from their initial precursor by the presence of both LC3 and Rab7 marker proteins [Webber, 2010]. Whether autolysosomes contain mAtg9 is still unknown. Although this suggests that mAtg9 does not cycle back to its peripheral pools, evidence indicates that this could nevertheless occur under certain conditions [Young, 2006].

6.2: The function of mAtg9

Although the exact functions of mAtg9 remain to be demonstrated, few hypotheses have been formulated. As its yeast counterpart, mAtg9 is thought to be at least partially responsible for membrane delivery to the forming autophagosome because this protein is associated with lipid bilayers. On membranes, it is distributed asymmetrically and co-localizes with Bif-1, a membrane-binding protein [Tooze, 2010]. Bif-1 has a BAR domain through which it is able to induce membrane curvature. As discussed in Chapter 2, Atg9 is also involved in membrane tethering and function. Taking these considerations together, it can be concluded that mAtg9 plays a role in autophagosome architecture, possibly by regulating membrane tethering/fusion and phagophore elongation.

This notion is also supported by the fact that in cells where mAtg9 was knocked down using siRNA probes, autophagosomes are smaller than those present in wild type cells [Webber, 2010]. The

presence of, albeit small, autophagosomes is remarkable, since Atg9 deletion in yeast results in the total disruption of autophagosome biogenesis. This can be explained either by siRNA efficiency, or because recently, evidence regarding an alternative Atg9-independent pathway has been obtained [Webber, 2010]. How this pathway exactly functions, especially without the sole postulated membrane-delivering protein mAtg9, remains to be elucidated.

6.3: The anterograde transport of mAtg9

Currently, only 1 binding partner for mAtg9 has been identified in high eukaryotes, the p38 interacting protein (p38IP) that was initially not classified as an Atg protein [Webber, 2010]. In cells where p38IP was knocked down, mAtg9 appears to be constricted under starvation conditions, indicating that p38IP is required for starvation-induced trafficking of mAtg9 [Webber, 2010].

The p38IP-mAtg9 interaction is regulated by the p38α mitogen-activated protein kinase (MAPK). Absence of p38α results in an increased amount of mAtg9-positive autophagosomes, indicating that p38α is a negative regulator of autophagy [Webber, 2010]. For a currently unknown reason, however, p38α is not essential for autophagy in various tissues. The p38α kinase activity is active in presence of nutrients [Casas-Terradellas, 2008]. Thus, it acts as a nutrient sensor and when activated, p38α becomes phosphorylated and in turn this modification leads to a dissociation of the p38IP-mAtg9 interaction [Yang, 2010].

The p38α competes with mAtg9 for p38IP binding [Webber, 2010]. Non-phosphorylated, inactive p38α has a lower affinity than mAtg9 for p38IP. In contrast, when p38α becomes phosphorylated, its affinity for p38IP is higher than that for mAtg9. Activation and inactivation of p38α occurs under rich medium and starvation conditions, respectively. Thus, the following model has been proposed [Webber, 2010]: In rich medium, p38IP is bound to the phosphorylated form of p38, while mAtg9 cycles between the TGN and its endosomal pools. Upon starvation, p38α becomes quickly dephosphorylated, disrupting the p38α-p38IP interaction. The consequent binding between p38IP

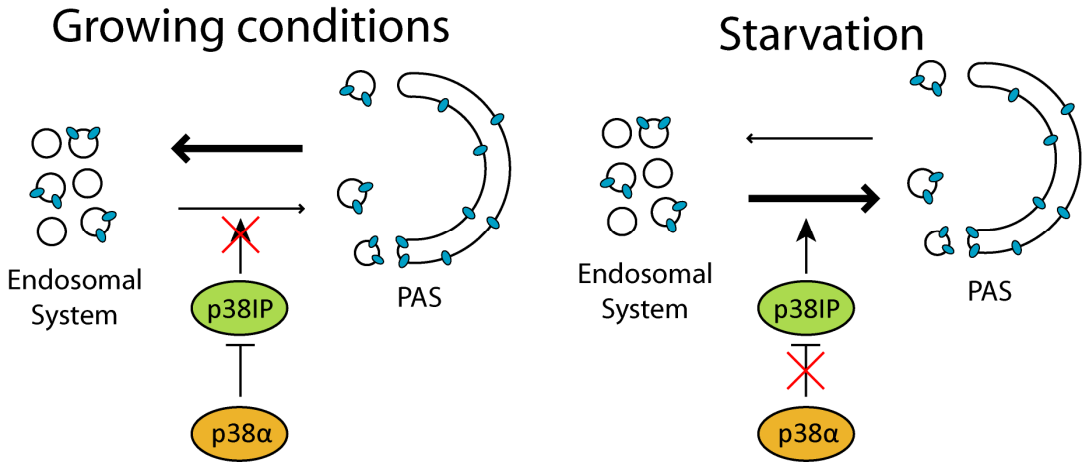

Figure 9. mAtg9 trafficking in growing and starvation conditions. During growing conditions, mAtg9 (blue) cycles between the endosomal system and the TGN (not pictured), with minimal transport to the PAS. Atg9 trafficking regulation by pI38P is inhibited by p38α, which is activated by the presence of amino acids. During starvation, p38α is inactivated, which allows dephosphorylated p38IP to interact with mAtg9 and facilitate its transport to the PAS.

and mAtg9 causes the complex to traffic to the PAS, where autophagosome formation is induced (Figure 9).

Other proteins that play a role in anterograde transport in yeast Atg9, e.g. Atg11, Atg23 and Atg27, have no known orthologues or homologues in mammals. Thus, mAtg9 anterograde transport is either facilitated in a different way than in yeast, or unknown proteins have a similar role to that of the yeast factors.

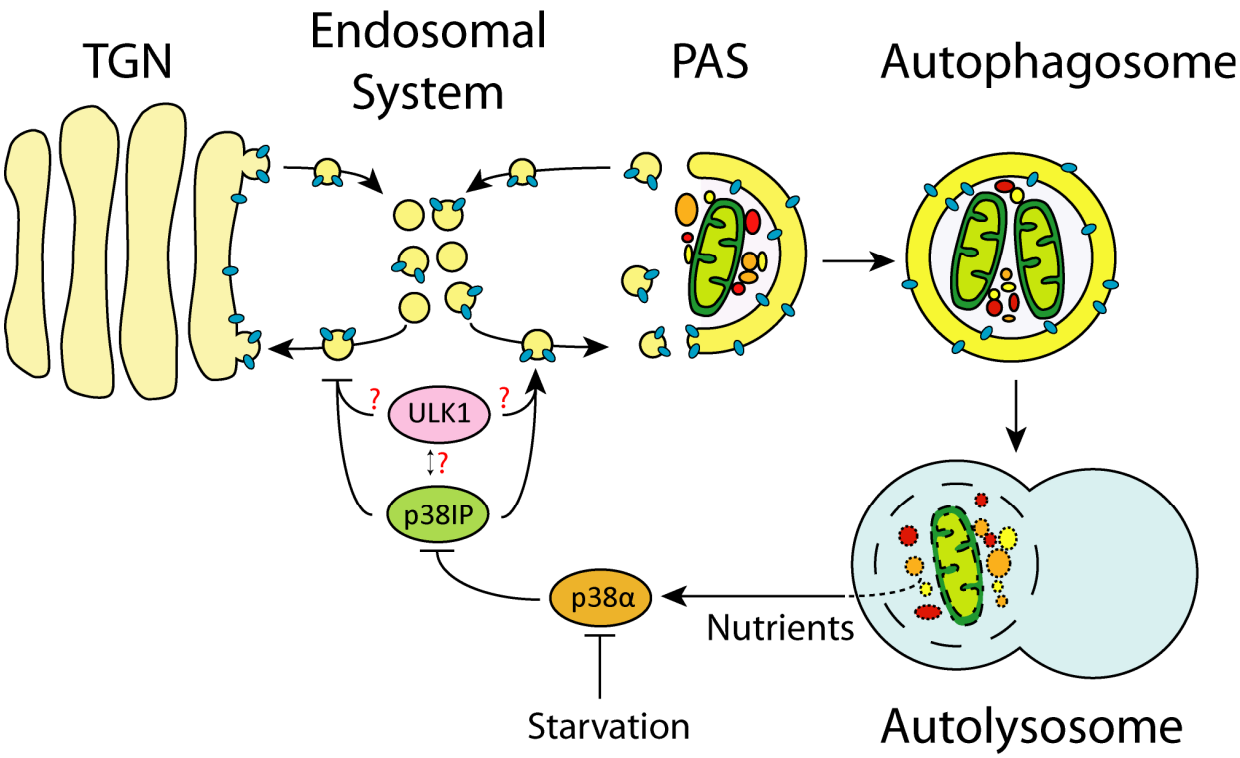

Figure 10. Model for mAtg9 cycling. mAtg9 cycles between the TGN and the endosomal system. When p38α is deactivated (for example due to starvation), p38IP becomes active and induces mAtg9 anterograde transport from the endosomal system to the PAS, which eventually results in autophagosome formation. Degradation of cytoplasm in the autolysosome results in the generation of nutrients such as amino acids which activate p38α, resulting in an inhibition of p38IP and anterograde mAtg9 transport. ULK-1 also influences mAtg9 transport, possibly by interacting with p38IP, but the mechanisms behind this are currently unknown.

6.4: The retrograde transport of mAtg9

Little is known about the mechanism mediating mAg9 retrograde transport. Since only one binding partner has been discovered, it is highly possible that there are additional unknown proteins that regulate the trafficking of mAtg9 to and from its peripheral pools. p38IP, however, may be responsible not only for the anterograde trafficking, but also for the retrograde transport of mAtg9. A possible hypothesis that could explain this notion is that mAtg9 has the strong characteristic of localizing to its peripheral pools and p38IP may overcome this tendency and translocates mAtg9 to the PAS. When nutrients are added, p38 is activated and competitively binds p38IP, disrupting the p38IP-mAtg9 interaction and causing mAtg9 to regain its tendency to localize to the peripheral pools. This type of regulation could also represent a crucial feedback mechanism to protect the cell from

death by starvation-induced autophagy, since autophagy facilitates the degradation of abundant organelles, resulting in free amino acids that can re-activate p38, which in turn binds to p38IP, thus impairing mAtg9 anterograde transport with an inhibitory effect on autophagy. In this manner, overactivation is prevented (Figure 10).

Recently, other mammalian homologues of yeast Atg proteins involved in the retrograde transport of Atg9 have been investigated and it appears that they are also (partly) responsible for mAtg9 cycling in high eukaryotes as well.

Interestingly, the absence of ULK1/mAtg1/Unc-51 [Mizushima, 2010], leads to an accumulation of mAtg9 at a juxta-nuclear compartment that is thought to be the PAS [Young, 2006]. This suggests that ULK1 acts as its yeast counterpart, regulating the retrograde transport of Atg9 [Chen, 2009].

One of the mammalian counterparts of Atg18, WIPI-1, might also regulates mAtg9 cycling. Research has shown that the PtdIns3P-phosphatase Jumpy is a negative regulator of autophagy, because overexpression of Jumpy causes an accumulation of mAtg9 at the PAS while Jumpy knock-down resulted in an increase in the autophagosome number [Vergne, 2009]. Since WIPI-1 associates with PtdIns3P, a hypothesis that can explain the possible role for WIPI-1 is that Jumpy action depletes the PtdIns3P pool at the PAS [Matsunaga, 2010], eliminating the WIPI-1-PtdIns3P association and thus WIPI-1 recruitment to the PAS. PtdIns3-associated WIPI-1 would induce retrograde transport of mAtg9. Further research, however, is required to confirm this hypothesis.

CHAPTER 7: PRACTICAL WORK

7.1: Vps13-Atg18 interaction

7.1.1 Introduction

One of the core Atg proteins is Atg18 [Obara, 2008]. As mentioned in Chapter 5, Atg18 is required for retrograde transport of Atg9 and therefore for correct progression of autophagy. Atg18 is not only found on autophagosomes, but also on endosomes. At this location, it exhibits a PtdIns3P effector function, which is required for endosomal membrane recycling from the vacuole [Dove, 2004]. One of the Atg18 binding partners in autophagy is Atg2 [Obara, 2008]. Recently, it has been hypothesized that during recycling from endosomal membranes Atg18 might interact with Vps13, a protein of unknown function, with a sequence partly similar to Atg2. If this interaction is true, then Atg18 might be the effector protein while its binding partners (Atg2 and Vps13) determine whether Atg18 exhibits its function on autophagosomes-to-PAS or on endosome trafficking. In this experiment, we aimed to investigate whether Atg18 binds to Vps13.

7.1.2. Approach

Cells were grown to 1 OD_{600} in 50 ml of YPD (1% yeast extract, 2% peptone, 2% glucose), collected by centrifugation and resuspended in 1 ml of ice-cold lysis buffer [20 mM Tris-HCl, pH 8.0, 150 mM KCl, 5 mM $MgCl_2$, 1% Triton X-100], supplemented with 10 µl of 100 µM phenylmethylsulfonyl fluoride (PMSF) and 20 µl of 50 mM protease inhibitor cocktail (Roche). Next, cells were lysed using glass beads and vortexing (2x for 1 min) and centrifuged at 13'000 rpm for 10 min at 4°C. For the total lysate, 75 µl of supernatant were taken. For the affinity isolation, the rest of the supernatant was incubated for 2h at 4°C with 50 µl of IgG beads (GE Healthcare, Waukesha, U.S.), supplemented with 20 µl of 100 µM PMSF and subsequently washed 4 times with 1 ml of lysis buffer and centrifuged at 1'200 rpm for 3 min. Most of the supernatant was removed and the remaining liquid aspirated away using a 27G¾ syringe. Then, the immuno-isolated proteins were eluted from the beads with 75 µl SDS-PAGE sample buffer and incubated at 100°C for 5 min. Finally, 15 µl of eluates were separated on a 5% or 10% SDS-PAGE gel before being analyzed by immuno-blotting using anti-PA and anti-HA antibodies, respectively.

Table 2. Strains used in this experiment:

SEY6210 Vsp13-PA*::Trp1*	control for an unspecific binding to IgG beads
SEY6210 *Vps13-PA::Trp1 Atg18-3xHA::HIS5*	to check Vsp13-Atg18 interaction
SEY6210 *Vsp13-PA::Trp1 Atg21-3xHA::HIS5*	to check Vsp13-Atg21 interaction
SEY6210 *Vsp13-PA::Trp1 Hsv2-3xHA::HIS5*	to check Vsp13-Hsv2 interaction
SEY6210 *Atg18-3xHA::HIS5*	control for unspecific binding to IgG beads
SEY6210 *Atg21-3xHA::HIS5*	control for unspecific binding to IgG beads
SEY6210 *Hsv2-3xHA::HIS5*	control for unspecific binding to IgG beads
SEY6210 *Atg18-3xHA::HIS5 Atg2-PA::Trp1*	positive control for the pull-down

7.1.3 Results and discussion

Immuno-blotting for total lysate confirmed the presence of Atg18 and its homologues Atg21 and Hsv2 in the correct samples (Figure 11, second gel from the top). Vsp13 and Atg2 were not detected (Figure 11, first gel from the top) and this might be explained because the cellular levels of these proteins are very low. All the PA fusion proteins were found in the immuno-isolates confirmed a correct execution of the experiment (Figure 11, third gel from the top). The immuno-isolates confirmed the interaction between Atg18 and Atg2 (Figure 11, bottom gel), which has previously been shown [Obara, 2008] and was used as a positive control for the pull-down experiments. Weak interactions are visible between Atg18 and Vps13, and Atg21 and Vps13, but these are unspecific interactions, since they can also be observed in the control pull-downs. Hsv2 does not interact with Vps13, either, since no band can be observed in the Vps13-Hsv2 pull-down. Taken together, these data suggest that Vps13 does not interact with Atg18 or either one of its homologues.

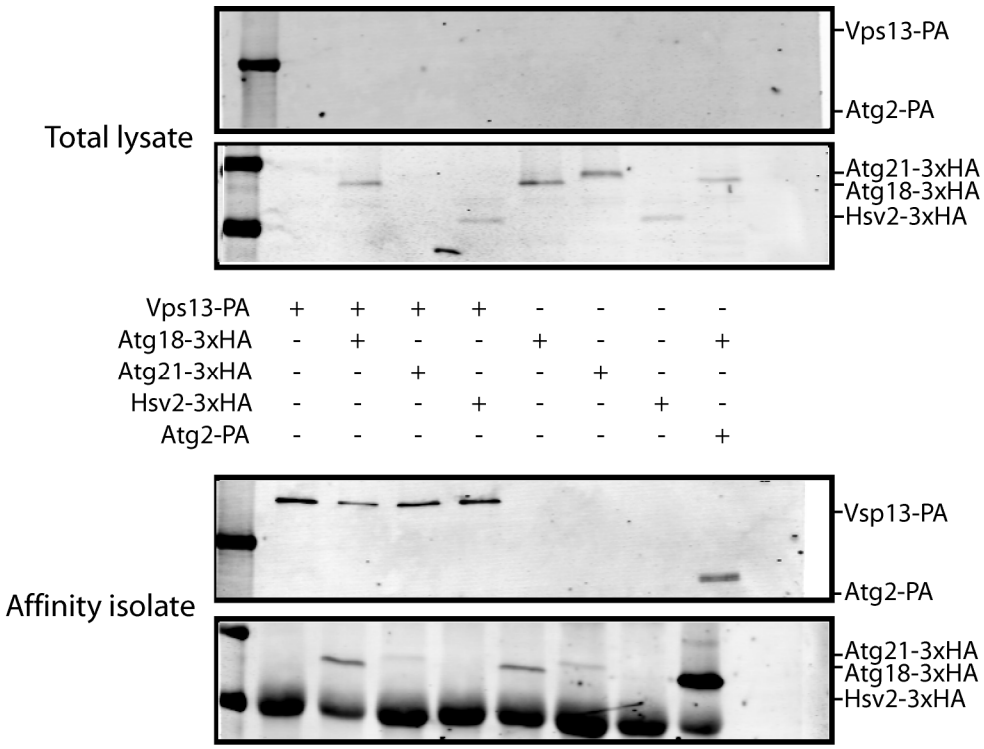

Figure 11. The Vps13 Atg18 interaction. HA-tagged Atg18 interaction with PA-tagged Vps13 was explored by a pull-down. Samples, total extracts and immuno-isolates, were prepared by SDS-PAGE and analyzed by Western blot using anti-PA and anti-HA antibodies. All HA-tagged proteins could be observed in the total lysate (*first gel from the top*), indicating that these proteins were indeed present in the yeast strains. The PA-tagged proteins, however, could not be detected (*second gel from the top*). All the PA- fusions were detected in the immuno-isolates (*third gel from the top*), Atg18-3xHA was pulled-down by Atg2-PA (*bottom gel, line 8*) confirming the binding between these two proteins. Atg18 and Atg21 appeared to be pull-down by Vsp13 (*bottom gel, lines 2 and 3*) but these are unspecific interactions since similar bands can also be observed for the control pull-downs (*bottom gel, lane 5 and 6*).

7.2: Effects of Atg18 loop mutants on mitophagy and pexophagy

7.2.1: *Introduction*

Atg18 contains an N-terminal WD40 domain that is predicted to fold into a seven-bladed β-barrel. The seven surfaces of the β-barrel are connected by six amino acid loops, among which loop (lp) 1, lp2 and lp5. Preliminary results in the laboratory indicate that these loops might play a role in binding other proteins and are therefore interesting to investigate. Our lab has previously studied the effects of mutating charged amino acid residues within lp1, lp2 and lp5 on the function of Atg18 in two types of selective autophagy, pexophagy and mitophagy. However, results were inconsistent and therefore, we decided to repeat one more time these experiments. For their realization, we have looked at the degradation of two proteins, Pex14 and OM45, that are present on peroxisomes and mitochondria, respectively. Both these proteins were chromosomally tagged with GFP at their C-terminus in a yeast strain in which Atg18 was deleted. Each of the different Atg18 loop mutants was introduced on a plasmid. In wild type cells, upon first inducing the proliferation of these organelles and then triggering their degradation by autophagy (nitrogen starvation), the cellular levels of Pex14-GFP or OM45-GFP decrease. However, since GFP is relatively stable in the vacuole, this decrease is accompanied by an increase of free GFP, as opposed to a yeast strain in which autophagy is not functional. The OM45-GFP processing assay and the pexophagy assay were conducted as previously described [Kanki, 2009; Hutchins, 1999].

7.2.2: *Approach*

In short, yeast was grown in media to induce the formation of mitochondria or peroxisomes. The cells were then shifted to starvation medium (SD-N; 0.17% yeast nitrogen base without amino acids, 2% glucose) to induce autophagy and collected by centrifugation after 0h and 6h. The cell pellet was then resuspended in 500 µl of ice-cold 10% tri-chloroacetic acid (TCA). Samples were incubated on ice for 30 min and then centrifuged at 13'000 rpm for 5 min. Supernatants were removed and the pellet resuspended in 1 ml of ice-cold acetone by sonication and put on ice. Samples were again centrifuged (13'000 rpm for 5 min) and the supernatant discarded. The pellet was dried at room temperature and subsequently glass beads and 75 µl of Sample Buffer were added before resuspension by sonication. Next, samples were vortexed for 1 min, heated at 95°C and centrifuged at 14'000 g for 1 min. Finally, samples were separated on a 12% SDS-PAGE gel and analyzed by immuno-blotting using anti-GFP, or anti-GFP and anti-Fox3 antibodies for mitophagy and pexophagy, respectively.

Table 3. Strains used in this experiment

SEY6210 *promOM45-GFP::TRP1 atg1Δ::HIS5*	negative control for autophagy, since Atg1 is a core Atg protein
SEY6210 *promOM45-GFP::TRP1*	WT; positive control for autophagy
SEY6210 *promOM45-GFP::TRP1 atg18Δ::HIS5*	in this strain, Atg18 WT or the loop mutants were introduced on a plasmid
SEY6210 *promPex14-GFP::TRP1 atg1Δ::HIS5*	negative control
SEY6210 *promPex14-GFP::TRP1*	WT; positive control for autophagy
SEY6210 *promPex14-GFP::TRP1 atg18Δ::HIS5*	in this strain, Atg18 WT or the loop mutants were introduced on a plasmid

7.2.3: *Results and discussion*

In the mitophagy assay (Figure 12), immuno-blotting for GFP confirmed the presence of OM45-GFP in all samples at least at the 0h time point. Induction of autophagy appears to function correctly, since the relative amount of OM-45-GFP decreased over time in WT strain (Figure 12, *lanes 2 and 4*), while the amount of free GFP increased. In the negative control *Δatg1* and as expected, free GFP did not accumulate over time (Figure 12*, lanes 1 and 3*). In the complete *Atg18* knock-out (Figure 12*, lanes 5 and 6*), the relative amount of free GFP also did not increase, confirming that Atg18 is also essential for mitophagy. In the *Atg18* loop mutant strains, it appears that the relative amount of free GFP in contrast to OM45-GFP, did not increase over time in the *Δatg18-lp1*, *Δatg18-lp2*, and *Δatg18-lp1,2* strains (Figure 12*, lanes 7, 8, 9, 10, 11 and 12*), indicating that these loop mutants are sufficient to disrupt Atg18 function. In the *Δatg18-lp5* strain (Figure 12*, lane 13 and 14)*, it appears that the amount of free GFP slightly increases, which would imply that this mutation does not completely hamper the function of Atg18. Further research, however, is required to confirm these data, since the increase of free GFP in the loop 5 mutant is not significant.

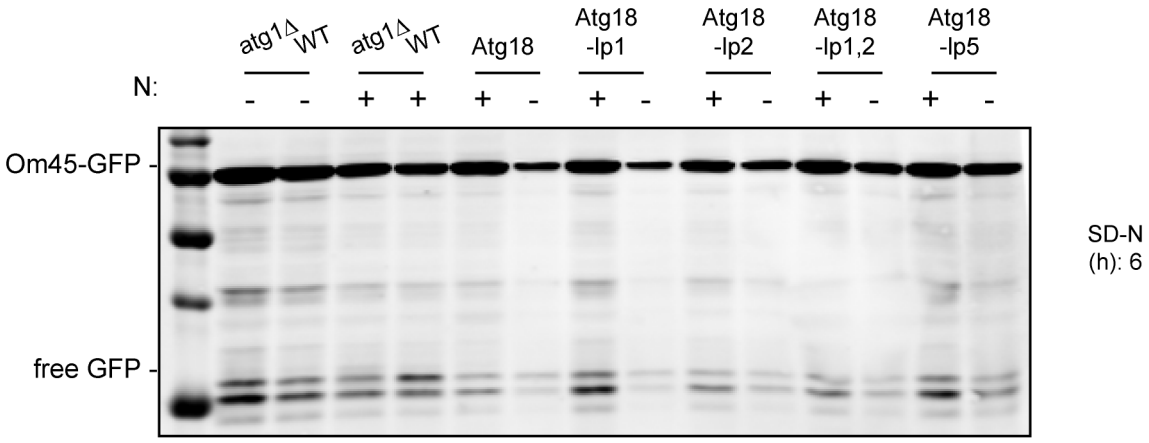

Figure 12. The effects of loop-mutations on Atg18 functioning in mitophagy. The amount of OM45-GFP compared with free GFP in several Atg18 loop-mutants was investigated through SDS-PAGE preparations and subsequent Western blot analysis using anti-GFP antibodies. Samples were taken at 0h (+) and 6h (-). In the *WT* strain (*lane 4*), the amount of OM45-GFP compared with free GFP was lower than in the control (*group 2, lane 2*), indicating that mitophagy was functioning correctly. This was confirmed by the *Δatg1* strain (*lane 3*), in which the ratio free GFP:OM45-GFP was relatively lower than in the control (*lane 1)*. The amount of OM45-GFP in the *Δatg18lp1/2/1,2/5* strains (*lanes 5, 7, 9, 11 and 13*) was relatively higher than the control groups (*lanes 6, 8, 10, 12 and 14*). The amount of free GFP was higher as well in these groups, implying that the Atg18 loop mutants do not have any effect on the efficiency of mitophagy. However, since the *Δatg18* strain also displayed the same effect on mitophagy, our results remain inconclusive.

In pexophagy (Figure 13), the relative amounts of Pex14-GFP were compared with those of free GFP. Fox3, a peroxisomal protein, was also used for the same analysis. No significant amount of free GFP is visible in any of the lanes. Since Pex14-GFP bands are visible, this phenomenon cannot be ascribed to defective antibodies. A possible explanation that might explain the lack of free GFP is that induction of peroxisomal formation is complicated and requires a certain amount of time. A suggestion for improvement would therefore be to lengthen both the time for peroxisome induction, as well as pexophagy, or to expose cells to a higher dose of peroxisome-inducing compounds such as oleic acid [Guan,J. 2001]. To monitor pexophagy, we also looked at the degradation of Fox3, a peroxisomal protein. In this case, a specific antibody against Fox3 was used. Unfortunately, we were unable to detect any bands by Western blot, which could be due to the antibody or the level of expression of Fox3. This remains to be tested in the future.

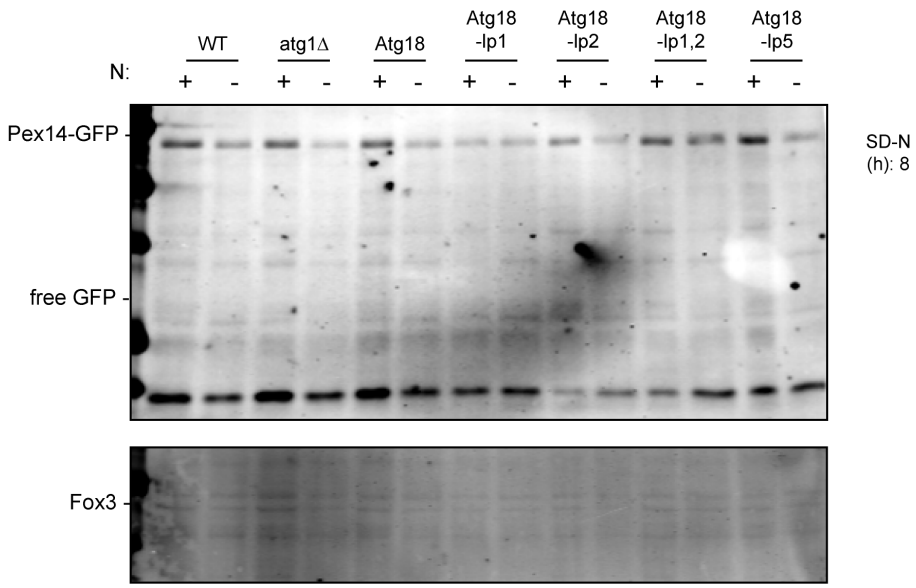

Figure 13. The effects of loop mutations on Atg18 functioning in pexophagy. The shift in free GFP:Pex14-GFP ratio was explored through SDS-PAGE and subsequent analysis by Western blot using anti-GFP antibodies. Samples were taken before (+) and 8h after (-) autophagy induction. No significant amount of free GFP could be observed in all the samples. Fox3 was also used to monitor pexophagy.

7.3: Atg18 ubiquitination

7.3.1: *Introduction*

Recent data in the laboratory indicates that Atg18 could undergo post-translational modifications, since a slightly higher band of Atg18 could be observed in specific experiments. Two examples of post-translational modifications are ubiquitination and phosphorylation. In this experiment, we investigate whether Atg18 is ubiquitinylated and if the introduced mutations in loop 1 have any effect on this.

7.3.2: *Approach*

Yeast cells were grown in rich (YPD; 1% yeast extract, 2% peptone, 2% glucose) or synthetic minimal media (SMD; 0.67% yeast nitrogen base, 2% glucose, amino acids and vitamins as needed). A similar pull-down experiment was performed as described in Chapter 7.1.2. Finally, 15 µl of eluates were separated on a 5% or 10% SDS-PAGE gel before being analyzed by immuno-blotting using anti-PA, anti-Myc and anti-UB antibodies.

Table 4. Strains used in this experiment

SEY6210 *ATG2-PA::TRP1 atg18Δ::HIS5*	control for unspecific binding to IgG beads
SEY6210 *ATG2-PA::TRP1 atg18Δ::HIS5*	in this strain, Atg18-13xMyc or Atg18 lp1-13xMyc is introduced on a plasmid
SEY6210 *atg18Δ::HIS5*	in this strain, Atg18-13xMyc or Atg18 lp1-13xMyc is introduced on a plasmid

7.3.3: *Results and discussion*

Immuno-blotting analysis of one of the total lysates confirmed the presence of Atg18-13xMyc (Figure 14, *lane 2*). Atg2-PA could not be detected, presumably because of its low cellular levels. The anti-UB antibody results in the detection of a large number of ubiquitinylated proteins. Therefore, we decided to only load one input sample, since the input sample of the Atg2-PA-only strain is not of great importance for this experiment. The PA-fusion protein could not be detected in the first of the immuno-isolates (Figure 14, *upper blot*). This can be explained because this strain did not grow very well. The next two lanes confirm the Atg2-Atg18 and Atg2-Atg18 lp1 interaction, respectively, because Atg18 or Atg18 lp1 bands (Figure 14, *middle blot*) are pulled-down by Atg2. This is confirmed by the Atg18-13xMyc-only control, because no Atg18 could be detected in absence of Atg2-PA in the experimental sample. Atg18-13xMyc partly binds a-specifically to the beads, since a band is visible in absence of Atg2-PA (Figure 14, *middle blot*). These results indicate that Atg2 interacts with both Atg18 and, to a lesser extent, Atg18 lp1. Ubiquitin could not be detected on the isolated Atg18-Myc constructs in any lane (Figure 14, *bottom blot*), demonstrating that Atg18 is not ubiquitinylated.

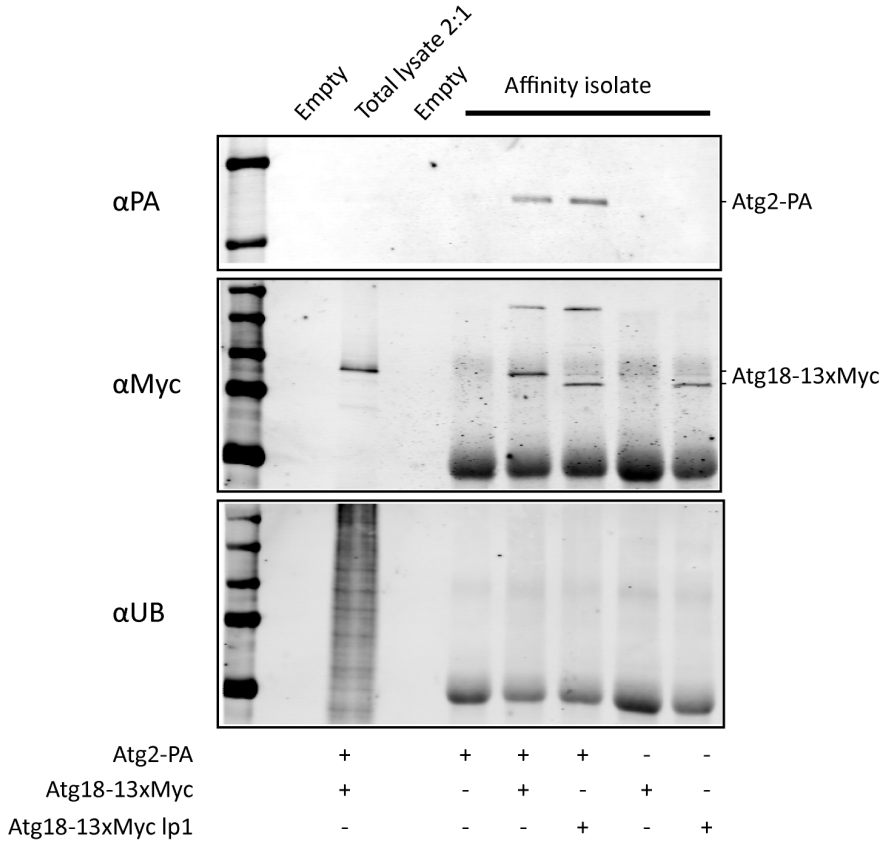

Figure 14: Atg18 ubiquitination. Ubiquitination of Atg18 and the effects of the *lp1* mutant on Atg2 binding were investigated through SDS-PAGE and Western blot analysis after pull-down experiments using antibodies for PA (*first gel from the top)*, Myc (*second gel from the top)* and ubiquitin (UB; *bottom gel*). Total lysates show the presence of Atg18-13xMyc. In the affinity isolates, the first lane should show the presence of Atg2-PA only. The second and third lanes show that Atg2-PA is pulled down with Atg18-13xMyc or Atg18-3xMyc lp1, respectively. Atg18-3xMyc binding with Atg2-PA is specific, since no band is visible in lane 4. A band is visible in lane 5 and this can be explained by the fact that Atg18 lp1 unspecifically binds to a certain extend to the beads. No ubiquitin bands are visible, indicating that Atg18 is not ubiquitinylated.

7.4: Phosphorylation of Atg18

7.4.1: *Introduction*

We have shown that Atg18 is not ubiquitinylated (Chapter 7.3). The question remains whether Atg18 is phosphorylated. In this experiment, the influence of the autophagy kinase Atg1 has been investigated, because it is the most likely candidate for Atg protein phosphorylation. We compared strains grown in rich media with strains starved for nitrogen, since the still unexplained shift might occur just in one of these two conditions. The influence of the mutations in *lp1* was also investigated, because the phosphorylation might be localized in this domain.

7.4.2: *Approach*

Cells were grown to 1 OD_{600} in 10 ml of rich medium (YPD; 1% yeast extract, 2% peptone, 2% glucose) and then shifted to starvation medium (SD-N; 0.17% yeast nitrogen base without amino acids, 2% glucose). Samples were collected during growing condition and after 2h of nitrogen starvation. 1 OD_{600} of cells was collected by centrifugation and TCA precipitated as described in Chapter 7.2.2. Then 15 µl of eluates were separated on a 10% SDS-PAGE gel before being analyzed by immuno-blotting using anti-Myc antibodies.

Table 5. Strains used in this experiment:

SEY6210 *atg18Δ::HIS5*	in this strain, Atg18-13xMyc or Atg18 lp1-13xMyc is introduced on a plasmid
SEY6210 *atg18Δ::HIS5 atg1Δ::TRP1*	in this strain, Atg18-13xMyc or Atg18 lp1-13xMyc is introduced on a plasmid

7.4.3: *Results and discussion*

Immuno-blotting revealed the presence of Atg18 in all strains (Figure 15). In the wild type cells (Figure 15, *lanes 1 and 2*) shows no shift in the strain in starvation conditions compared with the strain in growing conditions. The *atg1Δ* strain (Figure 15, *lanes 3 and 4*) shows a second band. Since Atg1 is absent, the shift cannot be explained by the hypothesis that Atg1 phosphorylates Atg18

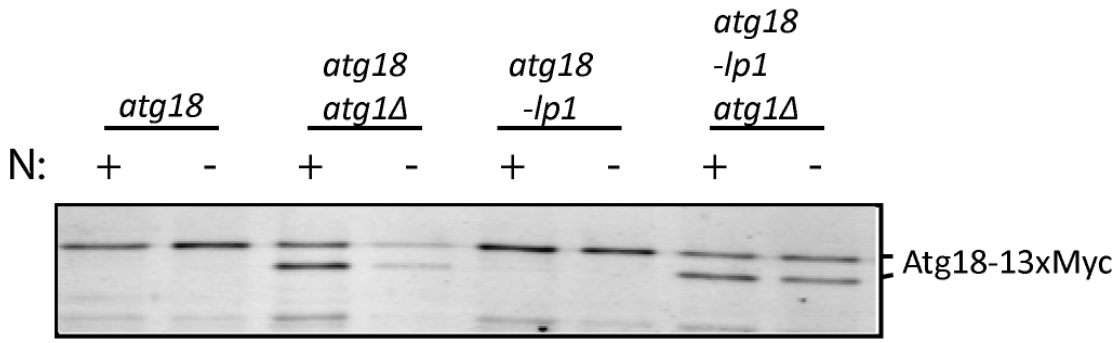

Figure 15: Atg18 phosphorylation. The phosphorylation of Atg18 by Atg1 was explored by a Western blot. In this blot, Atg18 (*lanes 1 and 2*), the *atg1* knock-out (*lanes 3 and 4*), the Atg18-lp1 mutant (*lanes 5 and 6)*, and the Atg18-lp1 mutant with *atg1* knock-out strain (*lanes 7 and 8*) were investigated.

because if this was true, the upper band should not have been present. In addition, no difference between growing and starvation conditions was observed. The analysis of Atg18 lp1 (Figure 15, *lanes 5 and 6*) gives a result similar to that of the wild type Atg18, showing 2 identical bands. This indicates that lp1 does not affect the hypothesized phosphorylation of Atg18. The *atg18-lp1 atg1Δ* strain (Figure 15, *lanes 7 and 8*) is similar to the *atg18-lp1 atg1Δ* strain, since the 2 Atg18-13xMyc bands are present in both growing and starvation conditions as well. Taken all together, these data suggest that Atg18 is not phosphorylated by Atg1 and the *lp1* mutant does not affect this post-translational modification.

7.5: Atg18 lp mutant and Atg2 expression

7.5.1: *Introduction*

In this experiment, our aim was to verify the expression levels of Atg18 lp mutants and Atg2 from constructs under the control of the GAL1-inducible promote*r*. Two strain backgrounds were used; a strain lacking *ATG18* (*atg18Δ)*, and a strain also lacking this gene but with *atg2* chromosomally tagged with 3xHA and the GAL1 promoter (ERY058). Both strains were transformed with plasmids containing either *ATG18* or one of the *ATG18* loop mutants under the control of the *GAL1* promoter.

7.5.2: *Approach*

Cells were grown to 1 OD_{600} in 10 ml of rich medium (YP-Galactose: 1% yeast extract, 2% peptone, 2% galactose). 1 OD_{600} of cells was collected by centrifugation and TCA precipitated as described in Chapter 7.2.2. Then 15 µl of eluates were separated on a 10% SDS-PAGE gel before being analyzed by immuno-blotting using anti-Myc antibodies.

Table 6. Strains used in this experiment

BY4247 *atg18Δ::kanMX4 HIS3MX6::pGAL1-3HA-ATG2-TAP::LEU2*
BY4247 *atg18Δ::kanMX4*
In both strains, Atg18 WT or each of the different Atg18 lp mutants (C-terminally tagged with 13xMyc) were introduced on a plasmid. On these plasmids, the expression of Atg18 was not under the control of its own promoter but the *GAL1* promoter.

7.5.3: *Results and discussion*

Immuno-blotting for Myc confirmed the presence of Atg18 in all strains (Figure 16), indicating that Atg18 is expressed adequately, although the Atg18 lp1,2 mutant had a slightly lower expression level (Figure 16, *lane 4*). Similar to these strains, the strains in which Atg2 was tagged with 3xHA, the Atg18 loop mutants are expressed. In the *atg18* lp5 strain (Figure 16, *lane 10*), the expression levels of both *atg18* loop5 and *atg2* are lower compared with the other strains but this could be due less cell extract loaded on the gel. Together, these data indicate that all strains express Atg18 loop mutants, although Atg18 lp1,2 in the *atg18Δ* strain, Atg2 in the Atg18 ERY058 strain, and Atg2 and Atg18 lp5 in the Atg18 lp5 ERY058 strain are expressed to a lower extent.

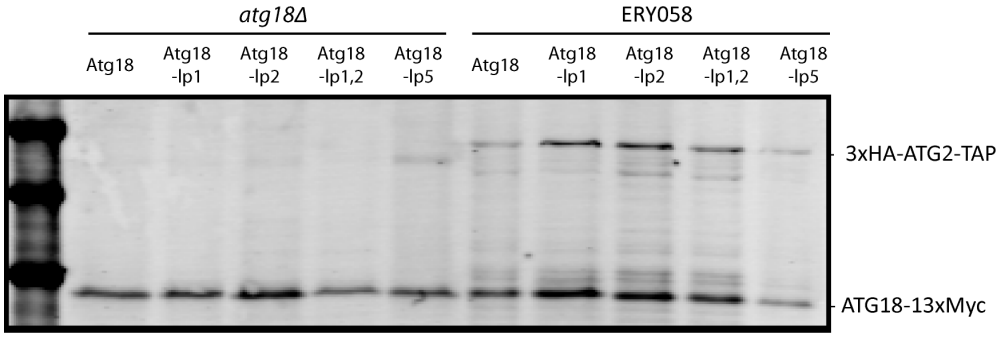

Figure 16: Atg18 and Atg2 expression. To explore the expression levels of the Atg18 loop mutants and Atg2 under the control of the GAL1 promoter, samples obtained from growing cells were separated by SDS-PAGE and analyzed by Western blot. Lanes 1 to 5 are strains in which *ATG18* was knocked-out and transformed with plasmids carrying either *ATG18* or *atg18* loop mutants. In all lanes, Atg18-13xMyc can be detected, although the expression levels for the lp1,2 mutant are slightly lower compared with the rest of the samples. In lanes 6 to 10, strains were not only transformed with *atg18* loop mutant plasmids, but *ATG2* was also chromosomally tagged with 3xHA and under the control of the *GAL1* promoter. Atg18-13xMyc is visible in all lanes, although it has a slightly smaller band in the Atg18 lp5 lane. Atg2-3xHA-TAP is visible in all lanes, but less abundantly in Atg18 and Atg18 lp5 strains.

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