Chapter 5

Screening for components of the Atg machinery involved in Atg9 trafficking

Susana Abreu and Fulvio Reggiori
Abstract
Atg9 is a highly conserved transmembrane protein essential for all types of macroautophagy (hereafter autophagy). In yeast, Atg9-containing vesicles are derived from the Golgi and shuttle between cytoplasmic pools and the site where autophagosomes are formed. This dynamic behaviour has led to propose that Atg9 may be an important regulator of autophagosome biogenesis. Some of the other key factors of autophagy, the autophagy-related (Atg) proteins, have been associated to Atg9 trafficking, but most of them remain to be investigated. Here we screened for the involvement of the Atg core machinery in Atg9 transport to and from the PAS during both the cytosol-to-vacuole targeting pathway, a selective type of autophagy, and bulk autophagy. Within this screen, we were able to confirm some of the previous findings but also unveil some new putative Atg proteins mediating a determined step in Atg9 trafficking under specific growth conditions.

Keywords
Atg9, Atg protein, phagophore assembly site, trafficking, autophagy, Cvt pathway

Introduction
Autophagy is a conserved and essential cellular process involved in the turnover of unwanted cellular components and invading pathogens. The hallmark of this pathway is the sequestration of the structures targeted to degradation, by double-membrane vesicles called autophagosomes. Autophagy operates at basal level under growing conditions to maintain cell homeostasis, but it can also be induced by stress conditions such as nutrient starvation to rapidly supply energy to the cell through the bulk degradation of the cellular components, including cytoplasm and organelles. In addition to bulk non-selective autophagy, cells possess selective types of autophagy where specific structures are exclusively sequestered into autophagosomes. The first selective type of autophagy to be discovered and one of the best-characterized ones, is the yeast cytosol-to-vacuole targeting (Cvt) pathway. This transport
route delivers large oligomers mainly formed by the vacuolar hydrolase aminopeptidase 1 into vacuoles\textsuperscript{2,3}. In yeast, autophagosomes are formed perivacuolarly at a specific site called the pre-autophagosomal structure or phagophore-assembly site (PAS). At this location, it is believed that an initial membrane cistern, named the phagophore or isolation membrane, is generated and elongates around the cellular components targeted for degradation to finally close into an autophagosome\textsuperscript{1,4}. Complete autophagosomes subsequently fuse with the vacuole releasing their content in the lumen of this organelle, where it is degraded by resident hydrolases\textsuperscript{1}. The molecular machinery mediating autophagy is composed by the so-called autophagy-related (Atg) proteins, and numerous of them have been uncovered with genetic screens in yeast \textit{Saccharomyces cerevisiae}. The biogenesis of autophagosomes is orchestrated through the action of a highly conserved core machinery, subdivided into five functional groups of Atg proteins: the Atg1 kinase complex, the autophagy-specific phosphatidylinositol 3-kinase (PI3K) complex, the Atg9 trafficking system, and the Atg8- and Atg12-ubiquitin conjugation systems\textsuperscript{5}. Atg9 is an integral protein with six conserved transmembrane domains essential for both autophagy and the Cvt pathway\textsuperscript{5–8}. In all organisms, Atg9 appear to principally localize to the trans-Golgi network (TGN) and endosomes, although it has also been detected at the plasma membrane and in vicinity of mitochondria\textsuperscript{9–11}. Atg9 buds off from the Golgi to form cytoplasmic Atg9-containing membranes, which comprise highly and low mobile 30-60 nm vesicles\textsuperscript{8,11,12}. Atg9 cycles between this location and the PAS\textsuperscript{13}, and because of this dynamics, it has been proposed that Atg9 contributes as one of the membrane sources for the formation of the phagophore and possibly its elongation\textsuperscript{12,14}. In yeast, an average of three Atg9-containing vesicles reaches the PAS and very likely participates to the formation of the phagophore through homo- and heterotypic fusion events\textsuperscript{12,14–16}. As Atg9 is detected on the external membrane of complete autophagosomes\textsuperscript{12,17}, this implies that it is probably retrieved during the fusion of these large vesicles with the vacuole or shortly afterwards as
it is not detected on the vacuole limiting membrane. In mammalian cells, in contrast, ATG9A-containing vesicles interact with but do not become incorporated into the autophagosomal membranes, and therefore it seems that ATG9A may be principally involved in the regulation of the phagophore expansion\textsuperscript{18}.

Trafficking of Atg9 has mainly studied in yeast. In this organism, the movement of Atg9 towards the PAS is mediated by different members of the autophagy core machinery: Atg11, Atg17, Atg23, Atg27\textsuperscript{12,13,19–24}. Atg23 and Atg27, which do not have mammalian homologues, very likely form a complex with Atg9\textsuperscript{25}. Atg23 and Atg27 thus display an identical similar distribution throughout the cytoplasm and to the PAS like Atg9, and their localization is mediated by Atg9\textsuperscript{13,26,27}. Both proteins are required for the Cvt pathway and efficient autophagy, and for Atg9 transport under both situations\textsuperscript{26,27}. More recently, evidences have been provided showing that Atg23 and Atg27 might participate in the biogenesis of Atg9-containing vesicles\textsuperscript{12}. Atg27 is additionally involved in retaining Atg9 in the correct compartments as in its absence, Atg9 gets mislocalized and degraded in the vacuole\textsuperscript{22}. Atg11 binds Atg9 directly, mediating its anterograde transport to the PAS in an actin-dependent manner during Cvt pathway\textsuperscript{19,23}. Atg11 functions as a link between the Cvt complex and the components that will promote the assembly of PAS, through a direct interaction with Atg19 (which interacts with Atg8) and Atg1\textsuperscript{28}. As Atg11 is involved in all the selective types of autophagy in yeast, including mitophagy and pexophagy, it is likely that this protein plays a key role is orchestrating the organization of the PAS in these contexts as well. Atg11 still plays a partial role in the Atg9 recruitment to the PAS under nutrient starvation conditions, but not essential to promote bulk autophagy\textsuperscript{23}. Under those conditions, the function of Atg11 in Atg9 transport to the PAS appears to be taken over by Atg17\textsuperscript{20}.

The Atg9-containing membranes retrieval from the PAS to their cytoplasmic pool requires the subunit of the Atg1, Atg13, the PI3K complex and the Atg9 binding partners Atg2 and Atg18. Omission of one of these proteins restricts Atg9 to the PAS during both Cvt pathway and Autophagy\textsuperscript{13,29}. Atg1 and Atg13 compose the Atg1 kinase complex,
which is involved in autophagosome biogenesis and mediate autophagy initiation in response to nutrient deprivation\textsuperscript{30}. Known Atg1 complex interactors such as Atg11 and Atg17, however, appear to not be implicated in Atg9 retrograde trafficking\textsuperscript{13}. The PI3K complex is responsible for the synthesis of phosphatidylinositol-3-phosphate on autophagosomal membranes and it is also involved in the Atg9 retrieval from the PAS\textsuperscript{13,31}. Atg9 is still partially retrieved from the PAS in cells lacking Atg14, the specific subunit of the autophagy-specific PI3K complex, possibly due to the mobilization to the PAS of phosphatidylinositol-3-phosphate synthesized by the endosome-specific PI3K complex\textsuperscript{13}. Atg2 and Atg18 are two interacting proteins that can associate to Atg9 and in their absence Atg9 concentrates to the PAS\textsuperscript{13,32}. Interestingly, Atg2 and Atg18 recruitment to the PAS depends on the Atg1-Atg13 and the PI3K complex, mechanistically linking all the factors that have been so far connected to Atg9 retrieval from the PAS\textsuperscript{32–34}.

In mammalian cells, ATG9A is distributed between the TGN and endosomes\textsuperscript{8}. During starvation ATG9A redistributes from the TGN towards a peripheral pool with an increased co-localization with autophagosomes through a mechanism that depends on the Atg1 homologue ULK1, ATG13 and the activity of the PI3K complex\textsuperscript{8,35}. The transport to autophagosomal membranes appears to principally occur at the recycling endosomes, where SNX18 recruits DYNAMIN2 to induce budding of ATG9A-positive membranes. In parallel, a TBC1D14-TRAPPII-like complex facilitates the trafficking of ATG9A from the recycling endosomes to Golgi, to maintain the ATG9A pool necessary for autophagy\textsuperscript{36,37}.

As an essential Atg protein regulating selective and non-selective types of autophagy, it is crucial to better understand Atg9 trafficking to gain better insights into its molecular function. In order to investigate whether other Atg proteins play a role in yeast Atg9 trafficking, we performed an imaging-based screen to identify new factors within the Atg machinery core machinery involved in the transport of Atg9 to and from the PAS. We identified different new putative factors involved in
this aspect of autophagy, which may operate under specific growing conditions.

**Results**

**Identification of Atg proteins involved in Atg9 transport to the PAS**

In order to investigate whether other members of the Atg machinery are involved in the movement of Atg9-containing membranes towards the PAS, we took advantage of the transport of Atg9 after knockout of *ATG1* (TAKA) assay. Atg1 is involved in the retrieval movement of Atg9 to its cytoplasmic pools and deletion of this protein causes Atg9 to accumulate and be restricted to the PAS. Knockout of a gene in an *atg1Δ* mutant background that results in the same phenotype as the original *atg1Δ* strain, i.e. a single perivacuolar Atg9-positive punctum, indicates that the ablated gene is not involved in the anterograde movement of Atg9 to the PAS. In contrast, Atg9 distribution in multiple cytoplasmic puncta in such double mutant shows that the deleted gene is involved in the re-localization of Atg9 from its cytoplasmic pools to the PAS.

![Figure 1. Atg9-GFP subcellular distribution under growing and nitrogen starvation conditions.](image)

<table>
<thead>
<tr>
<th>Growing conditions</th>
<th>Starvation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atg9-GFP</td>
<td>DIC</td>
</tr>
<tr>
<td>WT</td>
<td></td>
</tr>
<tr>
<td><em>atg1Δ</em></td>
<td></td>
</tr>
</tbody>
</table>

*Figure 1. Atg9-GFP subcellular distribution under growing and nitrogen starvation conditions.* Wild type (WT) and *atg1Δ* strains expressing Atg9-GFP (KTY097 and SAY001) were grown to an exponential growing phase in SMD medium before to be nitrogen starved in SD-N medium for 3 h. Cells were imaged before and after starvation. DIC, differential image contrast. Scale bar, 5 µm.
We examined Atg9 subcellular distribution in strains expressing endogenous Atg9-GFP lacking both ATG1 and the gene under analysis. Cells were imaged by fluorescence microscopy in both rich growing conditions and nitrogen starvation to determine the different requirements for Atg9 transport to the PAS during selective and non-

Figure 2. The role of Atg1 kinase complex subunits in the Atg9-GFP transport to the PAS using the TAKA assay. Subcellular distribution of Atg9-GFP in absence of both Atg1 and a subunit of the Atg1 kinase complex: Atg11 (SAY051), Atg13 (SAY053), Atg17 (SAY056), Atg29 (SAY057) and Atg31 (SAY058). DIC, differential image contrast. Scale bar, 5 µm.

We examined Atg9 subcellular distribution in strains expressing endogenous Atg9-GFP lacking both ATG1 and the gene under analysis. Cells were imaged by fluorescence microscopy in both rich growing conditions and nitrogen starvation to determine the different requirements for Atg9 transport to the PAS during selective and non-
selective types of autophagy, respectively. As expected\textsuperscript{13}, we observed Atg9-GFP distributed in several cytoplasmic puncta in the wild type (WT) strain and in a single perivacuolar dot in the \textit{atg1\Delta} background, during both the Cvt pathway, which operates under growing conditions in SMD medium and bulk autophagy triggered by nitrogen depletion in SD-N medium (\textbf{Figure 1}). Under, normal growing conditions and starvation, the deletion of genes of the Atg1 kinase complex like \textit{ATG13}, \textit{ATG17}, \textit{ATG29} and \textit{ATG31}, did not alter the concentrated Atg9 localization observed in the \textit{atg1\Delta} strain, except for the \textit{atg11\Delta} knockout (\textbf{Figure 2}).

In this background, Atg9-GFP had a similar subcellular localization as in the WT strain, i.e. it was distributed in several cytoplasmic puncta, in presence or absence of nitrogen, showing that this protein appears to be important for Atg9 trafficking during both selective and non-selective autophagy (\textbf{Figure 2}). The absence of two of the subunits of the PI3K complex, i.e. Atg6 or Atg14, did not affected Atg9 transport to the PAS in both tested conditions (\textbf{Figure 3}). Furthermore, we also found that deletion of \textit{ATG2} and \textit{ATG18} did not alter the accumulation of Atg9 in the \textit{atg1\Delta} background under conditions inducing selective autophagy.

\textbf{Figure 3}. Analysis of PI3K complex I components function in the Atg9-GFP transport to the PAS using the TAKA assay. Atg9-GFP localization in knockout strains for Atg1 and two of the subunits of the PI3K complex: Atg6 (SAY005) and Atg14 (SAY054). DIC, differential image contrast. Scale bar, 5 µm.
and non-selective autophagy (Figure 4). The knock-out of most of the elements of the Atg8- and Atg12- conjugation systems, i.e. ATG3, ATG4, ATG5, ATG7, ATG8, ATG10 or ATG16 in the atg1Δ deletant led to an Atg9 atg1Δ-like phenotype showing that those genes are not required for Atg9 anterograde transport (Figure 5). Simultaneous deletion of Atg1 and Atg12 under autophagy inducing conditions, however, resulted in Atg9 distribution in several puncta indicating that Atg12 may be involved in the transport of this protein under nitrogen deprivation (Figure 5).

Altogether, our data confirmed that Atg11 is involved in the Atg9 anterograde transport to the PAS and revealed that Atg12 could also have a similar function under autophagy-inducing conditions.

### Identification of the Atg proteins mediating Atg9 retrieval from the PAS

To screen for the Atg proteins that could be involved in the recycling of Atg9 from the PAS back to its peripheral locations, we analyzed the subcellular distribution of endogenous Atg9-GFP in cells lacking each one Atg genes.
Figure 5. Contribution of Atg8- or Atg12-conjugation system in the Atg9-GFP recruitment to the PAS using the TAKA assay. Subcellular distribution of Atg9-GFP in absence of both Atg1 and each one of the components of the conjugation system: Atg3 (SAY004), Atg4 (SAY050), Atg5, Atg7 (SAY006), Atg8 (SAY007), Atg10 (SAY008), Atg12 (SAY052) and Atg16 (SAY055). DIC, differential image contrast. Scale bar, 5 µm.
Once more, the fluorescence microscopy analyses were performed under conditions where the Cvt pathway and bulk autophagy operate. A deletion resulting in the same phenotype as \textit{atg1}\textDelta, a single Atg9-GFP-positive punctum per cell (Figure 1), indicates the possible involvement of that protein in the retrograde transport of Atg9\textsuperscript{13}. Contrarily, a gene deletion that produces an Atg9 cytoplasmic

\textbf{Figure 6. Involvement of Atg1 kinase complex subunits in Atg9 retrieval from the PAS.} Atg9-GFP subcellular distribution in absence of each one of the core components of the Atg1 kinase complex subunits: Atg11 (SAY042), Atg13 (SAY044), Atg17 (SAY047), Atg29 (SAY048) and Atg31 (SAY049). DIC, differential image contrast. Scale bar, 5 µm.
distribution in different puncta, does not indicate a functional association of that protein with Atg9 recycling from the PAS. We observed that the deletion of Atg1 kinase complex gene, \( ATG13 \), triggered the accumulation of Atg9 in one cell punctum, similarly to the \( ATG1 \) deletion, under selective and non-selective autophagy conditions. An analogous phenotype was observed upon the deletion of other members of the Atg1 kinase complex as Atg17, Atg29 (during Cvt pathway) and Atg31. On the other hand, \( atg11\Delta \) knockout strain presented a WT-like distribution of Atg9 in cell growing conditions (Figure 6).

![Figure 7](image)

**Figure 7. Examination of PI3K complex I subunits role during Atg9 recycling from the PAS.** The Atg9-GFP localization was performed in strains lacking two of the components of the PI3K complex: Atg6 (SAY122) and Atg14 (SAY045). DIC, differential image contrast. Scale bar, 5 µm.

In the absence of Atg14, Atg9 was limited to one fluorescent punctum during the Cvt pathway while during autophagy-inducing conditions this protein had an intermediate distribution phenotype, with the majority of Atg9 localized in one or two puncta (Figure 7). Deletion of \( ATG6 \), in contrast, displayed an Atg9 localization pattern like the WT during both Cvt pathway and autophagy (Figure 7). When analysing deletions of components of the Atg9 cycling system, we observed that
the knockout of ATG2, exclusively during autophagy, but not of Atg18, was constraining Atg9 to a single punctum per cell (Figure 8). Under Cvt pathway conditions, atg5Δ and atg7Δ cells displayed a phenotype identical to the one of the atg1Δ mutant, which in the case of the atg5Δ deletant was also observed during bulk autophagy. The absence of the other members of the Atg8- and Atg12- conjugation systems, contrarily, did not affect the WT distribution of Atg9-GFP except the deletion of ATG4, ATG10 or ATG16, which during Cvt pathway, led to an intermediate phenotype, where most of the cells exhibited one or two Atg9-GFP puncta per cell (Figure 8). Altogether, our results highlight the possible involvement of Atg5 and Atg7, but possibly also some of the other components of the two autophagy-specific ubiquitin-like conjugation systems, in Atg9 recycling from the PAS under selective type of autophagy.

![Figure 8](image_url)

**Figure 8. The role of the members of the Atg9 cycling system in Atg9 movement from the PAS.** Subcellular distribution of Atg9-GFP in absence of the factors involved in its cycling: Atg2 (SAY118), Atg18 (FRY469). DIC, differential image contrast. Scale bar, 5 µm.
Discussion
In both yeast and mammals, Atg9 shuttles between its cytoplasmic vesicle pool and the PAS to promote autophagosomal biogenesis and different Atg proteins are mediating this trafficking. In our work, we have applied a systematic approach to analyse the involvement of each core Atg protein in this aspect of autophagy. We identified factors possibly involved in the Atg9 transport to and from the PAS. Although some of them were already known, others have never been linked before to this function (Figure 10).

Atg proteins involved in the Atg9 anterograde transport to the PAS
Different studies have shown that Atg11 plays an essential role in Atg9 movement to the PAS during both the Cvt pathway and autophagy\textsuperscript{19,24,32}. In our study, we have confirmed that Atg11 functions in Atg9 trafficking to the PAS in both conditions (Figure 10). Atg13 has been described to be involved in the anterograde Atg9 transport during autophagy\textsuperscript{23}. However, our work showed no role of Atg13 in the anterograde movement of Atg9 to the PAS in agreement with another work\textsuperscript{13}. Although Atg17 was previously shown to be a factor essential for Atg9 anterograde movement to the PAS during both Cvt pathway and autophagy\textsuperscript{32}, we did not observe this involvement. This apparent discrepancy could be due to the different strategy used by the authors, which localized Atg9-GFP in a strain lacking simultaneously Atg2, Atg11 and Atg17. As Atg11 is involved in the anterograde Atg9 movement, the triple knockout strain is expected to display a block in Atg9 transport to the PAS. We present evidences for a possible role of Atg12 in Atg9 anterograde transport during autophagy (Figure 10). This function of Atg12 is very likely independent from its role that ultimately leads to Atg8 conjugation onto the autophagosomes. As the knockout of any other component of the Atg12- or Atg8-conjugation system appears to not affect Atg9 localization. The role of Atg12 in Atg9 trafficking may depend on an interaction between these two proteins. Direct binding between Atg9 and Atg12 has not been reported to date, but a genetic screen on yeast lifespan revealed a possible functional interaction...
between these two proteins\textsuperscript{39}. Moreover, a high-throughput screen aimed at identifying yeast protein complexes has revealed a possible interaction between Atg12 and Atg11, which in turn physically interact with Atg9\textsuperscript{40}.

**Atg proteins involved in the Atg9 retrograde transport from the PAS**

Different studies have shown that Atg1 participates in Atg9 retrograde transport from the PAS during both selective and non-selective autophagy\textsuperscript{13,19,25,32}, something that we also observed. Our results are in agreement with the previous findings that showed an Atg13 role in Atg9 recycling for the PAS during the Cvt pathway (Figure 10)\textsuperscript{13}. Here, we highlight a similar function of Atg13 under nitrogen starvation conditions as well. The Atg13-Atg9 complex mediates Atg9 anterograde transport during autophagy\textsuperscript{23}, but the possible involvement of this interaction in Atg9 recycling has not been explored. Here we also demonstrated that two other subunits of the Atg1 complex, i.e. Atg29 and Atg31, play a relevant role in Atg9 recycling from the PAS. Atg29 appears to have a function exclusively during the Cvt pathway, while Atg31 seems to be important under all tested conditions (Figure 10). As previously reported, Atg17 is not required for Atg9 retrieval from the PAS\textsuperscript{13}. Therefore, Atg29 and Atg31 are involved in Atg9 trafficking independently of the Atg17-Atg29-Atg31 complex. However, the lack of any of these proteins could have different consequences in the initial PAS organization, making the results complex in their interpretation. Our results confirm that Atg9 retrograde transport requires Atg14 during Cvt pathway\textsuperscript{13} and suggest a possible involvement of this protein also during autophagy (Figure 10). Atg14 physically interacts with Atg9\textsuperscript{41} and this interaction could mediate Atg9 transport from the PAS.

Surprisingly, Atg6, another subunit of the PI3K complex I, does not appear to be relevant for Atg9 trafficking and consequently Atg9 retrieval may be Atg14-dependent and not requiring PI3K as previously thought. Atg2 was shown to participate in the retrograde transport of Atg9 during the Cvt pathway\textsuperscript{13,32}. We confirmed this result and showed
that this involvement also holds true during autophagy-inducing conditions. The binding of Atg9 to Atg2 is probably essential on this trafficking process, and Atg1-dependent recruitment of Atg2 to the PAS was proposed as a possible explanation for Atg9 to be restricted to the PAS upon deletion of \( ATG1 \). On the other hand, Atg18 was shown to participate in Atg9 recycling towards the cytoplasmic pool during autophagy, but we could not confirm this observation in our study. There are conflicting results regarding Atg18 role in Atg2 recruitment to the PAS and therefore a more in-depth analysis would be important to determine Atg18 importance in the Atg9 trafficking in connection with Atg2.

In our screen, we found that some elements of the Atg8- and Atg12-conjugation systems, namely Atg5 and Atg7, are participating in the Atg9 retrograde trafficking, during autophagy and Cvt pathway, respectively. Contrary to the members of the other Atg functional groups, the absence of Atg5, Atg7 or any of the elements of Atg8- and Atg12-conjugation system, do not influence Atg2 recruitment to the PAS, suggesting that Atg5 and Atg7, like Atg2, could be the direct regulators of Atg9 retrograde trafficking. Moreover, Atg5 and Atg7 impede Atg8 to be recruited to the PAS, but \( ATG8 \) deletion does not interfere with Atg9 trafficking. Therefore, it is likely that Atg5 and Atg7 carry out a function in Atg9 recycling that is independent from their role in Atg8 conjugation. Taken in account our results, it is important to study the possibility of a physical interaction between Atg9, and Atg5 and Atg7. Although the Cvt pathway and autophagy share most of the Atg machinery involved in the Atg9 trafficking, some of the examined proteins appear to be specifically required for Atg9 movement during specific conditions. For example, Atg12 is required for Atg9 anterograde transport during autophagy. Moreover, Atg7 and Atg5 play a relevant function in Atg9 retrograde traffic during the Cvt pathway and autophagy, respectively.
Figure 9. Contribution of the Atg8- or Atg12-conjugation system elements in Atg9 retrieval from the PAS. Imaging of cells lacking the components of the Atg8- or Atg12-conjugation system: Atg3 (SAY067), Atg4 (SAY002), Atg5 (SAY120), Atg7 (SAY124), Atg8 (SAY125), Atg10 (SAY127), Atg12 (SAY043) and Atg16 (SAY046). DIC, differential image contrast. Scale bar, 5 µm.
Transcriptional regulation or post-translation modifications could influence the participation of each of these proteins in each autophagic process and explain the observed differences. One possible scenario could be that the proteins involved in either Cvt pathway or autophagy, provide the necessary specificities for Atg9 trafficking and consequently reflect on the individual properties of each pathway. For example, larger double-membrane vesicles are formed upon autophagy induction compared to those generated under nutrient rich conditions during the Cvt pathway, and ATG8 gene upregulation has been linked to this increase in size\textsuperscript{46,47}. On the other hand, the rate of autophagosome formation appears to be linked to Atg9 expression levels and this is thought to be associated with the possible involvement of Atg9 in the curvature and closure of the phagophore\textsuperscript{14,48}. The Atg proteins mediating Atg9 trafficking could for instance fine-tune Atg9 transport to and from the PAS, to modulate the formation of double-membrane vesicles of diverse size, with different cargo specificity. The further investigation of each one of these components will help to decipher how Atg9 controls the Cvt pathway and autophagy.

**Figure 10. Model for Atg9 trafficking in yeast.** During the Cvt pathway, Atg9 movement to the PAS requires Atg11. Atg9 recycling from the PAS involves Atg1, Atg2, Atg7, Atg13, Atg14, Atg29 and Atg31. Upon induction of bulk autophagy by
Materials and Methods

Yeast strains

The *S. cerevisiae* strains used in this study are listed in Table 1. Genes were knocked-out by homologous recombination using PCR fragments amplified with primers containing 60 bases identical to the flanking regions of the gene open reading frames\textsuperscript{49,50}, and were replaced with *URA3* or *LEU2* from *K. lactis*, *TRP1* from *S. cerevisiae* or *HIS5* from *S. pombe*. *ATG9* was tagged on its chromosomal locus by integration of GFP at the 3’end. The PCR product used for the integration was obtained by PCR amplification of the *GFP* and the *TRP1* marker sequences using pFA6a-GFP(S65T)-TRP1 as template plasmid\textsuperscript{49}. The expression of tagged Atg9-GFP was analysed by light microscopy. Gene disruptions were confirmed by PCR analysis.

Media

Cells were grown in rich medium (YPD; 1% yeast extract, 2% peptone, and 2% glucose) or synthetic minimal medium (SMD; 0.67% yeast nitrogen base, 2% glucose, and amino acids and vitamins as needed). To induce autophagy, cells were grown to a logarithmic (log) phase in YPD or SMD medium, and then transferred into a medium lacking nitrogen (SD-N; 0.17% yeast nitrogen base without amino acids and ammonium sulfate, and 2% glucose) for 3 h.

Fluorescence microscopy

Fluorescence signals were visualized with a DeltaVision RT fluorescence microscope (Applied Precision) equipped with a CoolSNAP HQ camera (Photometrix). Images were generated by collecting a stack of 20 pictures with focal planes 0.20 μm apart to cover the entire volume of a yeast cell and subsequently deconvolved using the SoftWoRx software (Applied Precision).
Chapter 5

Acknowledgements
The authors thank Daniel Klionsky for reagents. F.R. is supported by ALW Open Program (822.02.014), DFG-NWO cooperation (DN82-303), SNF Sinergia (CRSI13_154421), and ZonMW VICI (016.130.606) grants. S.A. is a recipient of a FCT Fellowship (SFRH/BD/95013/2013).

References
Chapter 5


44. Obara, K., Sekito, T., Niimi, K. & Ohsumi, Y. The Atg18-Atg2 complex


### Supplementary Table 1. Yeast strains used in this work

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRY469</td>
<td>SEY6210 $ATG9$::$TRP1$ atg18::$HIS5$</td>
<td>This study</td>
</tr>
<tr>
<td>KYT97</td>
<td>SEY6210 $ATG9$::$TRP1$</td>
<td>47</td>
</tr>
<tr>
<td>QYY030</td>
<td>SEY6210 $ATG9$::$TRP1$ atg1Δ::$URA3$ atg5Δ::$HIS5$</td>
<td>This study</td>
</tr>
<tr>
<td>SAY001</td>
<td>SEY6210 $ATG9$::$TRP1$ atg1Δ::$URA3$</td>
<td>This study</td>
</tr>
<tr>
<td>SAY002</td>
<td>SEY6210 $ATG9$::$TRP1$ atg4Δ::$URA3$</td>
<td>This study</td>
</tr>
<tr>
<td>SAY003</td>
<td>SEY6210 $ATG9$::$TRP1$ atg1Δ::$URA3$ atg2Δ::$HIS5$</td>
<td>This study</td>
</tr>
<tr>
<td>SAY004</td>
<td>SEY6210 $ATG9$::$TRP1$ atg1Δ::$URA3$ atg3Δ::$HIS5$</td>
<td>This study</td>
</tr>
<tr>
<td>SAY005</td>
<td>SEY6210 $ATG9$::$TRP1$ atg1Δ::$URA3$ atg6Δ::$HIS5$</td>
<td>This study</td>
</tr>
<tr>
<td>SAY006</td>
<td>SEY6210 $ATG9$::$TRP1$ atg1Δ::$URA3$ atg7Δ::$HIS5$</td>
<td>This study</td>
</tr>
<tr>
<td>SAY007</td>
<td>SEY6210 $ATG9$::$TRP1$ atg1Δ::$URA3$ atg8Δ::$HIS5$</td>
<td>This study</td>
</tr>
<tr>
<td>SAY008</td>
<td>SEY6210 $ATG9$::$TRP1$ atg1Δ::$URA3$ atg10Δ::$HIS5$</td>
<td>This study</td>
</tr>
<tr>
<td>SAY009</td>
<td>SEY6210 $ATG9$::$TRP1$ atg1Δ::$URA3$ atg18Δ::$HIS5$</td>
<td>This study</td>
</tr>
<tr>
<td>SAY042</td>
<td>SEY6210 $ATG9$::$TRP1$ atg11Δ::$HIS5$</td>
<td>This study</td>
</tr>
<tr>
<td>SAY043</td>
<td>SEY6210 $ATG9$::$TRP1$ atg12Δ::$HIS5$</td>
<td>This study</td>
</tr>
<tr>
<td>SAY044</td>
<td>SEY6210 $ATG9$::$TRP1$ atg13Δ::$HIS5$</td>
<td>This study</td>
</tr>
<tr>
<td>SAY045</td>
<td>SEY6210 $ATG9$::$TRP1$ atg14Δ::$HIS5$</td>
<td>This study</td>
</tr>
<tr>
<td>SAY046</td>
<td>SEY6210 $ATG9$::$TRP1$ atg16Δ::$HIS5$</td>
<td>This study</td>
</tr>
<tr>
<td>SAY047</td>
<td>SEY6210 $ATG9$::$TRP1$ atg17Δ::$HIS5$</td>
<td>This study</td>
</tr>
<tr>
<td>SAY048</td>
<td>SEY6210 $ATG9$::$TRP1$ atg29Δ::$HIS5$</td>
<td>This study</td>
</tr>
<tr>
<td>SAY049</td>
<td>SEY6210 $ATG9$::$TRP1$ atg31Δ::$HIS5$</td>
<td>This study</td>
</tr>
<tr>
<td>SAY050</td>
<td>SEY6210 $ATG9$::$TRP1$ atg1Δ::$URA3$ atg4Δ::$HIS5$</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>SAY051</td>
<td>SEY6210 ATG9-GFP::TRP1 atg1Δ::URA3 atg11Δ::HIS5</td>
<td>This study</td>
</tr>
<tr>
<td>SAY052</td>
<td>SEY6210 ATG9-GFP::TRP1 atg1Δ::URA3 atg12Δ::HIS5</td>
<td>This study</td>
</tr>
<tr>
<td>SAY053</td>
<td>SEY6210 ATG9-GFP::TRP1 atg1Δ::URA3 atg13Δ::HIS5</td>
<td>This study</td>
</tr>
<tr>
<td>SAY054</td>
<td>SEY6210 ATG9-GFP::TRP1 atg1Δ::URA3 atg14Δ::HIS5</td>
<td>This study</td>
</tr>
<tr>
<td>SAY055</td>
<td>SEY6210 ATG9-GFP::TRP1 atg1Δ::URA3 atg16Δ::HIS5</td>
<td>This study</td>
</tr>
<tr>
<td>SAY056</td>
<td>SEY6210 ATG9-GFP::TRP1 atg1Δ::URA3 atg17Δ::HIS5</td>
<td>This study</td>
</tr>
<tr>
<td>SAY057</td>
<td>SEY6210 ATG9-GFP::TRP1 atg1Δ::URA3 atg29Δ::HIS5</td>
<td>This study</td>
</tr>
<tr>
<td>SAY058</td>
<td>SEY6210 ATG9-GFP::TRP1 atg1Δ::URA3 atg31Δ::HIS5</td>
<td>This study</td>
</tr>
<tr>
<td>SAY067</td>
<td>SEY6210 ATG9-GFP::TRP1 atg3Δ::HIS5</td>
<td>This study</td>
</tr>
<tr>
<td>SAY118</td>
<td>SEY6210 ATG9-GFP::TRP1 atg2Δ::HIS5</td>
<td>This study</td>
</tr>
<tr>
<td>SAY120</td>
<td>SEY6210 ATG9-GFP::TRP1 atg5Δ::HIS5</td>
<td>This study</td>
</tr>
<tr>
<td>SAY122</td>
<td>SEY6210 ATG9-GFP::TRP1 atg6Δ::HIS5</td>
<td>This study</td>
</tr>
<tr>
<td>SAY124</td>
<td>SEY6210 ATG9-GFP::TRP1 atg7Δ::HIS5</td>
<td>This study</td>
</tr>
<tr>
<td>SAY125</td>
<td>SEY6210 ATG9-GFP::TRP1 atg8Δ::HIS5</td>
<td>This study</td>
</tr>
<tr>
<td>SAY127</td>
<td>SEY6210 ATG9-GFP::TRP1 atg10Δ::HIS5</td>
<td>This study</td>
</tr>
<tr>
<td>SEY6210</td>
<td>MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 mel GAL</td>
<td>51</td>
</tr>
</tbody>
</table>