MASTERARBEIT

Titel der Masterarbeit
„Atg34 in Selective Autophagy“

Verfasserin
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angestrebter akademischer Grad
Master of Science (MSc)

Wien, 2013

Studienkennzahl lt. Studienblatt:  B 066 866
Studienrichtung lt. Studienblatt:  Biochemie und Molekulare Biomedizin
Betreuerin / Betreuer:  Professor Dr. Graham Warren
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1. Abstract

Autophagy is a widely conserved, important pathway that allows eukaryotic cells to degrade cellular components and reuse them as a source of energy. It is characterized by the formation of a double membrane-bound vesicle, called autophagosome. Autophagosome formation is a stepwise process starting with the formation of a double membrane structure called isolation membrane. The isolation membrane elongates around cytoplasmic cargo, eventually fully isolating a portion of the cytoplasm within an autophagosome. The outer membrane of the autophagosome subsequently fuses with lytic cellular compartments; such as the lysosome in mammals or the vacuole in yeast and fungi; where the inner membrane and the cargo are degraded. During selective autophagy cargo material is selectively enclosed within autophagosomes. So-called cargo-receptor proteins, that specifically recognize their cargo as well as Atg8-family proteins on the isolation membrane mediate this selectivity. One such cargo-receptor protein is Atg34, which functions under starvation conditions in selective autophagy in yeast. It contains a C-terminal Atg8 family-interacting motif that mediates the interaction with Atg8 on the isolation membrane. It also binds to α-mannosidase 1 (AMS1) and thereby ensures the sequestration of AMS1 within autophagosomes. In this thesis Atg8-Atg34-interactions on the membrane were studied using a reconstituted system containing giant unilamellar vesicles – findings of these experiments further support former reports that this interaction of Atg34 with Atg8 depends on the Atg8 family-interacting motif. Furthermore, according to complexation experiments conducted with analytical size-exclusion chromatography, this interaction requires high protein concentrations (up to 740µM) of both proteins. In accordance with these findings no compelling evidence for any interaction between Atg34 and Atg8 was in a series of GST-pulldown assays where low concentrations (12.5µM) were used.
2. Introduction

Cells and organisms are constantly exposed to various stresses. Therefore, it is not surprising that organisms have evolved sophisticated mechanisms to alleviate these harmful impacts. In recent years, it has become clear that autophagy – a term derived from the Greek language meaning “eating oneself” [1, 2] – is a major pathway enabling cells to respond to different intracellular stress events. It represents an important lysosomal pathway that empowers cells to turn over cellular constituents, e.g., organelles, and reuse them as a source of energy [2]. Autophagy is triggered by certain types of stress, such as starvation, intracellular pathogens, accumulation of misfolded and aggregated proteins or dysfunctional organelles – such as mitochondria, peroxisomes, and the endoplasmic reticulum [2]. Consequently, autophagy has been implicated in the prevention of maladies such as cancer, neurodegenerative diseases (e.g., Morbus alzheimer, Morbus parkinson), cardiomyopathy, diabetes, liver disease, autoimmune diseases, and infections [2, 23].

Autophagy can be divided into three different types – known as macroautophagy (autophagy), microautophagy, and chaperone-mediated autophagy. While in all three types, cytosolic components are recognized [5] and transported into the lysosome [2], the transport mechanisms differ for each pathway [3] – implicated in figure 3. Additionally, the uptake and transport of the cargo-proteins can either be non-selective or selective [2, 3].

2.1 The Ubiquitin-Proteasome System vs. Autophagy

In eukaryotic cells, two principal mechanisms for protein degradation exist – the ubiquitin-proteasome pathway and the lysosomal pathway named autophagy [4]. In the ubiquitin-proteasome-system (UPS) protein degradation is carried out by a protease complex called the proteasome. The proteasome is formed by a large number of subunits (~50) and it exits in a variety of forms, all of them located in the cytoplasm and nucleus of eukaryotic cells. Proteasomes consist of a catalytic core to which one or two cap-complexes are bound. These caps regulate the catalytic activity of the whole complex. Proteins that are destined for degradation (e.g., misfolded or dysfunctional proteins) are therefore marked with one or more ubiquitin molecules; that result in its degradation by the proteasome. Conjugation of ubiquitin-molecules to these proteins via the UPS depends on E1 (ubiquitin-
activating enzyme), E2 (ubiquitin-conjugation enzyme) and E3 (ubiquitin-protein ligase) enzymes and is carried out in three steps. First E1 is activated in an ATP-dependent process in which an ubiquitin-molecule is added to the protein. Then the ubiquitin-molecule is transferred to a cysteine-residue in E2. And thirdly an isopeptide bond is established between the C-terminal ubiquitin on E2 and the 3-amino group of a lysine-residue in the target-protein. This last step is promoted by E3. Additional ubiquitin molecules are then conjugated to the first one creating a polymeric ubiquitin chain [1]. This ubiquitin tagging is necessary to ensure further processing via the proteasome. In the following regulatory subunits of the proteasome again remove the ubiquitin tag and facilitate the unfolding of the protein. Only unfolded proteins can enter the catalytic portion of the proteasome and thus be degraded [4].

There is evidence that autophagy and the UPS are not entirely separated protein degradation systems but function in a synergistic manner. There are cases when both pathways make use of the same substrate and regulatory proteins – for example α-synuclein can be broken down via macroautophagy, chaperone-mediated autophagy or the UPS [4].

2.2 Chaperone-mediated Autophagy
Chaperone-mediated autophagy (CMA) can be induced by physiological stress but a basal level can be observed too under normal cellular conditions. It is an important cellular quality control pathway for the removal of dysfunctional and misfolded proteins, adaptation to oxidative stress and starvation (recycling of non-essential proteins and organelles). It also plays a key role in the immune response (antigen presentation on MHC class II molecules) [4].

In CMA target proteins are specifically bound by certain heat-shock-proteins, which then facilitate the translocation of the cargo into the lysosome via the lysosomal-associated membrane protein 2A (LAMP-2A) [2, 3]. Thus CMA represents a highly selective form of autophagy, in which only one single soluble protein-substrate is sequestrated and degraded [3]. CMA so far has only been reported in mammalian cells [20].

In CMA protein structures that are not indispensable for cellular survival under critical conditions are selectively degraded to regain amino acids that can be recycled into essential proteins. Proteins that are targeted by CMA contain a
special amino acid sequence - the KFERQ-motif – which is normally hidden but becomes exposed and accessible when the proteins are unfolded. Saturability on the other hand occurs due to the usage of a receptor-protein (LAMP-2A) for protein-translocation into the lysosomal lumen. In CMA no autophagosomal vesicle is used for translocation [4, 25].

In CMA a complex of three different heat-shock-proteins including Hsc70, Hsc40 and Hsc90 is formed, that also contains Hsc70-interacting protein (Hip), Hsc70-Hsc90-organizing protein (Hop) and the Bcl2-associated anthanogene 1 protein (BAG-1). Hsc70 is a 73-kDa-protein, which belongs to the heat shock protein 70 family of chaperones [4]. Hsc70 interacts with soluble proteins by binding to their KFERQ-motif. The other proteins in the complex function as co-chaperones, mediating this interaction [3]. Substrate-binding to Hsc70 is an ATP-dependent process that facilitates the unfolding of target-proteins at the lysosomal membrane which is a prerequisite for their translocation into the lysosome [4]. The actual translocation across the lysosomal membrane requires the transmembrane-protein LAMP-2A, which enables this transport via four positive amino acids (KRHK/KHHH) at its cytoplasmic tail [5]. Substrate binding to LAMP-2A, induces multimerization in the otherwise monomeric protein. Multimerization facilitates substrate translocation alongside yet another chaperone protein, lys-Hsc70, which is located in the lysosomal lumen [3]. When the substrate enters the lysosome the LAMP-2A-multimere disassembles in a lys-Hsc70-dependent manner. Lys-Hsc70 is another limiting factor in the CMA-machinery, that can be saturated [4].

2.3 Microautophagy (MiA)

MiA is characterized by the direct uptake of cytosolic components by the lysosome (in mammals) or the vacuole (in fungi and plants) [3, 6]. During MiA the lysosomal or vacuolar membrane invaginates and buds off into the lumen. On the inside the the membrane bulges and forms the autophagic body, which then fuses with the vesicle and the enclosed cytosolic components can be degraded within the lysosome or vacuole (figure 1). The regulation and execution of MiA is still poorly understood [3, 6]. It is currently believed that MiA occurs in 5 sequential stages, consisting of 1. the invagination of the membrane and the formation of the autophagic tube, 2. vesicle formation, 3. the expansion of this vesicle, 4. scission
of the autophagic vesicle and 5. degradation of the vesicle and recycling of lysed components. These 5 stages of MiA are shown in figure 1. The invagination is believed to be driven by lipid-modifying proteins that produce spots on the membrane were transmembrane proteins are scarce and thus allowing for the membrane to deform. It has been shown that a dynamin-related GTPase named Vsp1p takes part in the regulation of the invagination. The formation of the Two ubiquitin-like (Ubl) conjugation systems take part in tube-organization. In the first one Atg7 – an E1-like enzyme – and Atg3 – an E2-like enzyme – conjugate Atg8 to the membrane-lipid phosphatidylethanolamine (PE). Cleavage of a C-terminal arginine-residue of Atg8 by the cysteine protease Atg4 is necessary. Via the second Ubl conjugation-system Atg7 and Atg10 – another E2-like enzyme – mediate the conjugation of Atg12 to Atg5. Atg12-Atg5 then binds to Atg16. This complex acts as an E3-like enzyme facilitating the conjugation of Atg8 to PE [6]. In yeast for further invagination of the autophagic body the vacuolar transporter co-chaperone (VTC) complex is also necessary. According to Li et al. 2012 [6] the second stage of MiA – vesicle formation – is believed to be caused by the scarcity of proteins in the tubular membrane and the high presence of lipids in these depleted parts. In the third stage a bubble like structure forms at the very top of the autophagic tube and expands in size. For the expansion and the following scission certain enzymes target the inside of the bubble [6]. MiA is regulated via signaling complexes such as the target of rapamycin (TOR, mTOR in mammals) and the exit from rapamycin-induced growth arrest complex (EGOC) [3, 6]. MiA can be induced via nitrogen-starvation or rapamycin-treatment. It presents an important cellular mechanism to balance the size of organelles, to keep membrane homeostasis and to help cells to overcome phases of nutrient scarcity [6].
Fig. 1: The 5 stages of MiA. Stage 1: Membrane invagination and formation of the autophagic tube. Stage 2: Vesicle formation and shaping of the autophagic body. Stage 3: Vesicle expansion. Stage 4: Budding of the vesicle and scission. Stage 5: Degradation of the vesicle and turn over of its cargo in the lumen of the lysosome/vacuole.

2.3.1 Selective Forms of MiA
Besides this non-selective form of MiA, three selective forms of MiA have been described. These include micropexophagy, piecemeal microautophagy of the nucleus (PMN) and micromitophagy. All three of them can be observed upon induction (by nitrogen-starvation or rapamycin treatment) in yeast. In micropexophagy dysfunctional peroxisomes are selectively enclosed by the vacuolar membrane. This is achieved by tentacle-like structures that emanate from the vacuolar membrane and encircle the peroxisome. Thus micropexophagy does not involve the formation of an autophagic tube. In PMN (figure 2) parts of the nucleus are degraded. This occurs via Velcro-like nucleus-vacuole-junctions and is regulated by TORC1 under starvation conditions [6, 26]. Lastly micromitophagy...
serves as degradation-process for mitochondria [27].

![Diagram of microautophagy](image)

Fig. 2: Piecemeal microautophagy of the nucleus. In stage 1 the membrane of the vacuole/lysosome and the nuclear envelope form contact sites via Velcro-like nucleus-vacuole-junctions. In stage 2 the vacuole/lysosome invaginates giving room for the junctions to enter into the lumen. In stage 3 a part of the nucleus buds off and the vacuole/lysosome encloses around it (stage 4). In stage 5 finally the micronucleus is released into the lumen for degradation.

### 2.4 Macroautophagy (MaA, Autophagy)

During macroautophagy (MaA, hereafter autophagy), intracellular cargo is delivered to the lysosome/vacuole in double membrane-bound vesicles, called autophagosomes [2, 3]. So far 36 different autophagy related genes (Atg) have been identified in yeast [10]. Most of them are conserved throughout plant and animal kingdoms. In contrast to CMA and MiA (discussed above), during
Autophagosomes are formed to isolate intracellular cargo from the cytoplasm. Autophagosomes are formed in a de novo manner. Initially, small membrane structures called isolation membranes (or phagophores) are observed. These structures elongate and finally their ends fuse giving rise to autophagosomes that enclose their cargo. After their formation autophagosomes fuse with lysosomes or the vacuole wherein their inner membrane and the cargo are degraded (see figure 3). Permeases and specialized transporters then transfer amino acids back into the cytosol where they can be reused. The membrane source for autophagosomes is still unclear. The endoplasmic reticulum (ER), the trans-Golgi, endosomes, mitochondria and the plasma membrane have all been suggested to serve as a membrane source [20]. In yeast autophagosome formation presumably occurs at the pre-autophagosomal structure (PAS). The PAS localizes in close proximity to the vacuole and it can be seen as punctuate structure, where most of the Atg-proteins localize [10]. For the assembly of the phagophore a set of various Atg-proteins is necessary, building the Atg1-ULK1 complex [2, 9, 28]. It is speculated that this complex, together with Atg2 and Atg18 [28], then recruits Atg9 – a transmembrane protein - which in turn recruits the lipids needed to form the isolation membrane [2, 9]. The exact function of Atg9 in autophagy are still to be elucidated [9, 10]. The initiation of phagophore formation is regulated by TOR-kinase. Under nutrient rich conditions TOR-kinase hyper-phosphorylates Atg13 and thereby inhibits the interaction of Atg13 with Atg1 [10, 28]. In mammals the initial processes of phagophore formation are even less understood. No PAS has yet been detected. Phagophores often appear to form in the proximity to the ER, the trans-Golgi or endosomes. Following the initial phagophore formation, elongation of the isolation membrane occurs through the interactions of Atg1 with 2 subunits of a class III PI3-kinase complex named vesicular protein sorting 34 and 15 respectively (Vps34 and Vps15) [9, 18]. Unlike other class III PI3-kinases Vps34 only uses phosphatidylinositol (PI) as a substrate to synthesize phosphatidyl-inositol-3-phosphate (PI3P). To generate PI3P Vps34 has to be activated by binding to Atg6 in yeast. After its generation PI3P facilitates autophagosome formation by recruiting further downstream signaling effectors [31].

Atg5 to Atg12 conjugation at the phagophore followed by Atg8-processing (microtubule-associated protein light chain 3 (LC3) in mammals [2]) are two other
important steps during autophagy, which are carried out by two distinct ubiquitin-like conjugation-systems. Atg8 is a ubiquitin-like protein that localizes to the PAS. It is originally synthesized in the cytosol comprising an arginine-residue at its C-terminus. This arginine-residue is removed by the cysteine protease Atg4 leaving a C-terminal glycine-residue, which is used as a substrate for a ubiquitin-like conjugation reaction mediated by Atg7 and Atg3. In this reaction Atg8 is conjugated to phosphatidylethanolamine (PE) in the isolation membrane [29].

For the conjugation of Atg5 to Atg12 Atg7 – an E1-like enzyme – is necessary. Atg7 binds to Atg12 via a glycine-residue at the C-terminus of Atg12. Atg12 is then transferred to Atg10 – an E2-like enzyme – which in turn establish a covalent link of Atg12 to Atg5 via a lysine-residue of Atg5. The Atg12-Atg5-conjugate then binds to an Atg16-dimer. Atg8-PE formation is facilitated by the Atg12-Atg5-Atg16-complex [2, 30]. Both Atg12-Atg5 and Atg8-conjugation systems are depicted in figure 4. It is believed that Atg8-PE mediates the tethering and hemifusion of liposomes [29]. Atg8 also acts as a receptor-protein for certain adaptor-molecules. After cargo-uptake and maturation the autophagosome fuses with the lysosome/vacuole to form an autolysosome [2].
Fig. 3: The characteristics of CMA, MiA and MaA at a glimpse. In CMA a chaperone complex binds misfolded cytosolic protein and facilitates its incorporation into the mammalian lysosome via Lamp-2A. MiA is characterized by the direct invagination of the lysosome/ vacuole, whilst in MaA a double membrane vesicle termed the autophagosome forms around the degradable cargo which finally fuses with the lysosome/ vacuole.
Fig. 4: The two ubiquitin-like conjugation systems involved in autophagosome formation. To form the Atg12-Atg5-Atg16 complex, Atg12 is conjugated to Atg5 via E1 and E2 like enzymes – Atg7 and Atg10, respectively. The Atg12-Atg5 conjugate then binds to an Atg16-dimer forming the Atg12-Atg5-Atg26 complex, that acts as an E3-like enzyme in the conjugation reaction of Atg8 to phosphatidylenolamine (PE) at the autophagosomal membrane. First the C-terminal arginine residue of cytosolic Atg8 is cleaved off by Atg4, then it is conjugated to PE via E1- (Atg7), E2- (Atg3) and E3-like enzymes.

2.5 Selective Types of Autophagy and the Cvt-Pathway
Although the core Atg-machinery is the same in non-selective and selective autophagy, selective autophagy requires additional mechanisms to selectively recognize cargo for sequestration into autophagosomes. To serve this purpose certain cargo-receptor-proteins have two conserved properties. Firstly they contain a certain structural motif that allows the selective recognition of cargo and secondly they interact with Atg8-family proteins on the isolation membrane [7, 21]. They thereby link the cargo to the growing autophagosomal membrane [7].
2.5.1 Other Forms of Selective Autophagy

Besides the already mentioned forms of selective autophagy (CMA, micropexophagy, piecemeal microautophagy of the nucleus (PMN) and micromitophagy) some other forms exist as well. In most of them the underlying molecular mechanisms still have to undergo extensive research and are not well understood yet. Among these forms are macromitophagy, macropexophagy, reticulophagy (degradation of ER), xenophagy (degradation of invading pathogens, such as viruses and bacteria) and ribophagy (ribosomes). Aggrephagy (degradation of intracellular protein-aggregates) is a form of selective autophagy restricted to mammals and is connected to neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's disease [7].

2.5.2 The Cvt-Pathway

The best understood form of selective autophagy is the cytoplasm-to-vacuole targeting (Cvt) pathway [7]. It is restricted to yeast but it is mechanistically analogous to other types of selective autophagy. It functions as a biosynthetic pathway to deliver three hydrolases, aminopeptidase 1 (prApe1) [17], α-mannosidase 1 (Ams1) [8, 9] and aminopeptidase 4 (Ape4) [32] – to the vacuole [8, 9, 32]. The Cvt-pathway makes use of the same core-machinery of the 17 Atg-proteins that also function during macroautophagy (shown in table 1). In addition the Cvt specific cargo-receptor-protein Atg19 and the adaptor-protein Atg11 are required [17]. The actin cytoskeleton seems to be an important factor too and is thought to be crucial for localizing the Cvt-vesicle and Atg9 to the PAS [10].

PrApe1 is a protein that – in its active form – hydrolyzes leucine-substrates. It is synthesized as a zymogen in the cytosol, where it forms large oligomeric complexes consisting of multiple prApe1-homododecamers. These oligomeric structures are essential for Atg19-binding [17] and together they build a large complex termed the Cvt-complex. In addition, Atg19 binds Ams1-oligomers and thereby incorporates them into the Cvt-complex. Upon specific Atg19-Atg11-interaction the Cvt-complex is then linked to the core Atg-machinery at the PAS. Atg11 interacts with the Atg1/13 kinase complex and Atg9 – and thereby ensuring the enclosure of the cargo by the growing isolation membrane [7, 10]. In the Cvt-pathway localization to the PAS also depends on the Vps53 tethering complex (VFT), consisting of Vps51, Vps52, Vps53 and Vps54 [33]. To ensure vesicle-
formation this complex acts together with Vps45 and the Q-SNAREs Tlg1 and Tlg2. Besides its interaction with Atg11, Atg19 also binds Atg8 via the LC3-interacting region (LIR), a motif also known as Atg8 family-interacting motif (AIM) [10, 22]. Atg8 is localizes to isolation membranes [8, 17]. Subsequently, a double membrane vesicle is formed around the Cvt-complex, termed the Cvt-vesicle. The outer membrane fuses with the vacuole to release the prApe1 cargo into the vacuolar lumen [7, 17]. Due to its similarity to autophagy in terms of the molecular machinery involved and its general molecular mechanisms, the Cvt-pathway is a valuable studying object to gain inside into the basic aspects of selective autophagy-processes in general [8]. A scheme of the cargo-uptake in the Cvt-pathway is depicted in figure 5.

Tab. 1: The 17 Atg-Proteins of the Core machinery and the 2 Additional Atg-Proteins of the Cvt-Pathway according to Lynch-Day and Klionsky, 2010

<table>
<thead>
<tr>
<th>Core-Machinery of Atg-Proteins</th>
<th>Additional Atg-Proteins in the Cvt-Pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atg1</td>
<td>Atg10</td>
</tr>
<tr>
<td>Atg2</td>
<td>Atg12</td>
</tr>
<tr>
<td>Atg3</td>
<td>Atg13</td>
</tr>
<tr>
<td>Atg4</td>
<td>Atg14</td>
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<td>Atg5</td>
<td>Atg15</td>
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<td>Atg6</td>
<td>Atg16</td>
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<td>Atg7</td>
<td>Atg18</td>
</tr>
<tr>
<td>Atg8</td>
<td>Atg22</td>
</tr>
<tr>
<td>Atg9</td>
<td>Atg11</td>
</tr>
<tr>
<td></td>
<td>Atg19</td>
</tr>
</tbody>
</table>
Fig. 5: Cargo-uptake in the Cvt-pathway. PrApe1 dodecamers homooligomerize to form oligododecamers. These are bound by Atg19 to form the Cvt-complex, into which Ams1 homooligomers are incorporated. Now Atg11 interacts via Atg19 with the Cvt-complex and actin filaments facilitating the binding to the PAS. At the PAS Atg19 interacts with Atg8 that is conjugated to the membrane ensuring the cargo-uptake into the double membrane Cvt-vesicle.

2.5.3 Cargo-Receptor-Protein Atg19 in the Cvt-Pathway

Cargo-receptor-proteins mediate selective autophagy by specific recognition of their cargo and simultaneous binding to Atg8 family proteins that localize on the developing autophagosomal membrane [17]. For the Cvt-pathway in yeast Atg19 is the cargo-receptor. Atg19 first binds to its cargo – prApe1-oligomers - to form the Cvt-complex into which Ams1-oligomers are incorporated. Then it interacts with Atg11 – the specific adaptor-protein that connects the cargo to the core-machinery at the PAS. And lastly it binds Atg8 at the PAS, ensuring that the membrane only expands around the Cvt-complex, thereby incorporating the cargo into the forming Cvt-vesicle [10].

Atg19 is a protein, composed of 415 amino acids that can be divided into 4
domains. The N-terminal domain (residues 1 – 123), the helical domain (residues 124 – 253), the Ams1-binding domain (192 - 387) and finally the C-terminal domain (residues 265 – 415). prApe1 binding is mediated by amino acids 153 – 191 that localize in the helical domain [8, 10]. Binding of prApe1 and Ams1 can occur simultaneously, since the two bonding sites are spatially separated on the Atg19-molecule [10, 11]. Using pulldown assays Watanabe et al. proposed that residues His-310 and/ or Glu-311 are necessary for Ams1-binding [8].

Upon interaction of Atg19 with Atg11 the Cvt-complex is tethered to the core Atg-machinery at the PAS. Atg11 specifically binds a motif at the C-terminus of Atg19. Atg11 itself is a large protein consisting of four coiled coil-domains. Amongst these it is the very last coiled coil-domain located on the C-terminus that binds Atg19. Other parts of Atg11 interact with Atg-proteins of the core-machinery (Atg1, Atg17, Atg20, or homo-oligomerisation) and two of its N-terminal domains (CC1 and CC2) are necessary for Atg9-binding [11].

2.5.3.1 Interaction of Atg19 with Atg8
At the PAS Atg19 selectively interacts with Atg8. Atg8 is a ubiquitin-like protein, that is conjugated to the membrane lipid phosphatidylethanolamine (PE) in the PAS. Atg8 is crucial for autophagosome or Cvt-vesicle formation and is thought to link the cargo-proteins to the isolation membranes. Atg8 comprises a C-terminal ubiquitin-like domain and a N-terminal helical domain. The N-terminal domain consists of 2 α-helices (α1 and α2) that are conserved in all Atg8-homologues and unique amongst ubiquitin-like proteins. The α-helices are followed by an exposed β-strand (β2, reaching from residues 48 to 52) – here, upon AIM/ LIR-binding a parallel intermolecular β-sheet is formed. Near β2 Atg8 has two hydrophobic pockets termed the W- and L-sites. These hydrophobic pockets are responsible for interacting with Trp- or Leu-residues in the AIM-/LIR-motif of Atg19. The AIM of Atg19 has been found in the C-terminus, where the WEEL-sequence - comprising residues 412 to 415 – interacts with Atg8. The WEEL-sequence forms the already mentioned intermolecular parallel β-sheet together with β2 of Atg8 by inserting Trp412 and Leu415 into the W- and L-sites of Atg8. This binding seems to be further facilitated by interactions through residues Glu413 and Glu414 of the WEEL-sequence with Arg67 and Arg28 of Atg8 [12].
2.5.3.2 Other Cargo-Receptor Proteins and Their Interaction with Atg8

Apart from Atg19 several other cargo-receptor-proteins have been characterized that act during different forms of selective autophagy. Amongst these PpAtg30 acts during pexophagy in *Pichia pastoris*, Atg32 during selective mitophagy in *Saccharomyces cerevisiae*, and p62/SQSTM1, NDP52, optineurin, NBR1, and Nix have been identified as cargo receptor proteins in mammalian cells. PpAtg30 is a peroxisome receptor, interacting with PpPex3 and PpPex4 at the peroxisome to transport it into the vacuole [34]. Atg32 - the cargo receptor protein for mitophagy – targets the mitochondrial membrane [35]. The mammalian cargo-receptor-proteins NBR1, optineurin, NDP52, p62/SQSTM1 [36] and Nix [37] incorporate ubiquitin-labeled proteins into autophagosomes. Besides PpAtg30 all of them share the WXXL-motif (LIR) that enables the interactions with Atg8, or one of the Atg8 family proteins in mammals [10, 17]. In 2010 Suzuki et al. reported the identification of yet another novel receptor-protein in *Saccharomyces cerevisiae* termed Atg34 [13].

2.5.3.3 The Cargo-Receptor Protein Atg34

Atg34 is a homologue of Atg19 and functions as a receptor for Ams1, incorporating Ams1 into the autophagosome via interactions with Atg11 and Atg8 [17]. Upon binding of Ams1 homo-oligomers a complex comparable to the Cvt-complex of preApe1-Atg19 is formed, and in this case is termed Ams1-complex [13]. While Atg19 is the prominent receptor-protein in the Cvt-pathway, Atg34 acts during the selective cargo uptake under starvation conditions where it functions redundantly with Atg19 during the transport of Ams1 into the vacuole. Like Atg19 Atg34 interacts with Ams1 via a C-terminal ABD. This interaction depends on amino acid residues 158 to 379. Additionally, it was shown, that residue His\(^{296}\) and Glu\(^{297}\) is essential for the binding of Ams1-oligomers. Structurally this ABD is comparable to the ABD of Atg19 (described above) and ABDs are highly conserved between these two proteins [8].

Atg34 too exhibits an Atg8 family-interacting motif, which is indispensable for Atg8-binding and crucial for tethering the Ams1-complex to the isolation membrane. The C-terminal AIM comprises the amino acids Trp\(^{409}\)-Glu\(^{410}\)-Glu\(^{411}\)-Ile\(^{412}\) [13]. Atg34 might be activated by phosphorylation, occurring under starvation conditions [13]. Differences in domain composition between Atg19 and Atg34 are shown in figure 6.
Fig. 6: Domain composition of Atg19 and Atg34 (not drawn to scale). Atg19 as well as Atg34 contain a C-terminal Atg8-interacting motif (AIM) and an Ams1-binding domain (ABD), but only Atg19 comprises a prApe1-binding site.
3. Rationale
The aim of this thesis was to elucidate the molecular basis of Atg34-Atg8-interactions and to create deeper insights into cargo-recruitment in selective autophagy. Therefore Atg34-Atg8-interactions as well as cargo recruitment were studied in solution and on artificial membranes (Giant Unilamellar Vesicles, GUVs).
4. Material and Methods

4.1 Generation of Bacterial mEGFP-Atg34 and mCherry-Atg34 Expression Constructs
Wildtype Atg34- and mutant Atg34W409A- (tryptophane in position 409 exchanged by alanin) constructs were prepared, consisting of N-terminal fluorescence tags (either mCherry or mEGFP-genes) plus an additional N-terminal histidin-tag to allow later affinity purification. The C-terminus, which contains the LIR-motif for Atg8-interaction remained free. Cloning was carried out in two steps first introducing the fluorescence-tag into the gene sequence followed by the introduction of the affinity-tag. A scheme of this cloning process is depicted in figure 8. Plasmid maps of the used vectors are shown in figure 8, 9 and 11.

Enzymes:
Polymerase:
Pfu-polymerase plus corresponding buffer (10x) (fermentas)
Restriction enzymes:
EcoRI-HF plus buffer Nr.4 (10x) (NEB)
BamHI-HF plus buffer Nr.4 (10x) (NEB)
NotI-HF plus buffer Nr.4 (10X) (NEB)
Ligase:
T4-ligase plus corresponding buffer (10x) (NEB)

Cell Lines:
Escherichia coli DH5α

Plasmids:
pmCherry C1
pmEGFP C1
pET Duet

Gel-electrophoresis:
Running buffer:
TAE (1x):
40mM Tris
20mM acetic acid
1mM EDTA

Agarose-gels:
0.5% Agarose in TAE (1x): 5g agarose dissolved in 100mL TAE (1%), addition of
5µL SYBR® Safe

Media:
LB (Luria Bertani)-Medium: Peptone 1%, yeast extract 0.5%, NaCl 1%

Antibiotics:
Kanamycin 50µg/mL
Ampicillin 50µg/mL

4.1.2 Introduction of the Fluorescence-Tag

4.1.2.1 Polymerase Chain Reaction
Standard PCR-methods were used. Template sequences of Atg34 and
Atg34W409A were contained in pGEX-4-T1. Primers (10µM) were ordered from
Sigma Aldrich, dNTPs (10µM) were from Fermentas. Primer sequences are
depicted in figure 7.

4.1.2.2 Purification of Insert-DNA
PCR-products were checked using gel-electrophoresis. Insert-DNA was then
purified using a Promega kit.

4.1.2.3 E. coli-Transformation with pmCherry C1 and pmEGFP C1
E. coli DH5α-cells were transformed with pmCherry C1- and pmEGFP C1-
plasmids. Plasmid uptake was forced by heat shock. To this end 50µL of cells were
mixed with 1µL of plasmid-DNA (~100ng plasmid-DNA) and kept on ice for 15
minutes, followed by a heat shock at 42°C for 1 minute. Then 450µL of LB-medium
were added and cells were incubated for 60 minutes at 37°C in a shaker at
600rpm. Cells were then pelleted by centrifuging at 3000rpm for 5 minutes. The
cell pellet was re-suspended and plated on LB-plates containing Kanamycin (50µg/mL). Plates were incubated over night. Single \textit{E. coli}-colonies were then picked and used to inoculate 15mL of LB-medium containing 50µg/mL Kanamycin. Cultures were grown over night.

\subsection*{4.1.2.4 Plasmid-Purification}
Over night cultures were centrifuged at 10.000rpm for 30s to collect the cell pellets. Plasmid purification was carried out using the MiniPrep Kit (Fermentas). The binding of plasmid-DNA was enhanced by incubating for 1-2min before spinning. To elute the bound plasmid-DNA distilled H$_2$O was used instead of elution buffer included in the MiniPrep Kit.

\subsection*{4.1.2.5 Restriction Digest}
With both plasmid- and insert-DNA a restriction digest was performed using restriction enzymes EcoRI-HF and BamHI-HF and buffer Nr. 4 (10x) (NEB). Restriction was carried out at 37°C for 2h. Success of the restriction digest was checked by Gel-electrophoresis. Cut plasmids were gel-purified using gel-electrophoresis. Both insert- and plasmid-DNA were Kit-purified (Promega).

\subsection*{4.1.2.6 Ligation}
Both wildtype Atg34 and mutant Atg34W409A were ligated with both pmCherry C1 and pmEGFP C1 plasmids using T4-Ligase (fermentas) and its corresponding buffer (10x). Ligation-mixes were incubated for 15min at room temperature.

\subsection*{4.1.2.7 \textit{E. coli}-Transformation with Ligated Constructs}
\textit{E. coli} DH5α-cells were transformed with plasmids pmCherry C1 containing either the open reading frame of the Atg34- or Atg34W409A-gene and pmEGFP C1 containing either the open reading frame of the Atg34- or Atg34W409A-gene. Transformation was carried by heat shock according to the above mentioned protocol. Over night cultures were again used for plasmid preparation (fermentas MiniPrep-Kit) as described before. With the purified plasmids a control digest was carried out and checked by gel-electrophoresis. Successful construct creation was additionally confirmed by DNA-sequencing (LGC Genomics, Sanger sequencing).
Fig. 7: Primer sequences used for DNA-amplification in the first cloning step. The mutation in the AIM-motif was introduced using a different reverse-primer.

5' (forward-primer)

CCCCCCCCGAATTC\text{\underline{\text{EcoRI}}}TATGAAAAATGC\text{\underline{G GTAGAAACG}}

3' (reverse-primer)

CCCCCCCCGGATCC\text{\underline{\text{BamHI}}}TTATTTTCTTCCCAAGTAAATGGC

3' (reverse-primer) W409A-mutant

CCCCCCCCGGATCC\text{\underline{\text{BamHI}}}TTATTTTCTTCC\text{\underline{C mutation}}CGCAGTAAATGG
Fig. 8: Plasmid map of pmCherry C1.
4.1.3 Introduction of the Affinity-Tag

4.1.3.1 Polymerase Chain Reaction
In the following step the former created constructs pmEGFP-Atg34, pmEGFP-Atg34W409A, mCherry-Atg34 and mCherry-Atg34W409A were amplified by standard PCR-methods. Furthermore a TEV-cleavage site was added for later protein purification purposes. Primer sequences are depicted in figure 10.

4.1.3.2 Purification of Insert-DNA
PCR-products were purified using gel-electrophoresis followed by Kit-purification (Promega).

4.1.3.3 *E. coli*-Transformation with pET Duet
Since pET Duet contains a histidin-sequence it was used to provide the affinity-tag
on the constructs N-termini, figure 11 shows a plasmid map of the pET Duet-1 vector. Again *E. coli*-DH5α-cells were used and plasmid uptake was forced by heat shock following the protocol mentioned. Ampicillin (50µg/mL) was added as selection marker.

4.1.3.4 Restriction Digest
With both plasmid- and construct-DNA a restriction digest was performed using restriction enzymes NotI-HF and BamHI-HF and buffer Nr. 4 (10x) all derived from NEB. Restriction was carried out at 37°C for 2h. Restricted plasmids were gel-purified using gel-electrophoresis. Both construct- and plasmid-DNA were Kit-purified (Promega). Success of the restriction digest was checked by Gel-electrophoresis.

4.1.3.5 Ligation of construct-DNA with pET Duet
Both mCherry- and mEGFP-Atg34 and mCherry- and mEGFP-Atg34W409A were ligated with pET Duet plasmids using T4-Ligase (fermentas) and its corresponding buffer (10x). Ligation-mixes were incubated for 15min at room temperature.

4.1.3.6 *E. coli*-Transformation with pET Duet containing either mCherry-Atg34, mEGFP-Atg34, mCherry-Atg34W409A or mEGFP-Atg34W409A
*E. coli*-DH5α-cells were used and plasmid uptake was forced by heat shock following the above mentioned protocol. Ampicillin (50µg/mL) was added as selection marker. Colonies of each plate were picked to inoculate 5mL of LB-medium containing Ampicillin (50µg/mL) as selection marker. Cultures were grown over night. Cell pellets were collected by centrifugation and plasmid-purification was carried out as mentioned above.

4.1.3.7 Control Restriction Digest
With both plasmid- and construct-DNA a restriction digest was performed using restriction enzymes NotI-HF and BamHI-HF and buffer Nr. 4 (10x) all derived from NEB. Restriction was carried out at 37°C for 1h and results were checked by gel-electrophoresis. To confirm that the cloning was successful, all samples were sequenced (LGC Genomics, Sanger sequencing).
Fig. 10: Primer sequences used for DNA-amplification in the second cloning step. BamHI- and NotI-restriction sites were introduced, as well as a TEV-cleavage site.

5′ (forward-primer)

```
CCCCCCCCGGATCCGGAAAAACCTGTATTTTCAGGGATGGTGAGCAAGGCGAGGAG
BamHI TEV-cleavage site
```

3′ (reverse-primer)

```
CCCCCCCCGCGGCCTTTATTTCTTCCAAGTAATG
NotI
```

3′ (reverse-primer) W409A-mutant

```
CCCCCCCCGCGGCCTTTATTTCTTCCAAGTAATGG
NotI
```
Fig. 11: Plasmid map of pET Duet 1.
Fig. 12: Scheme of the 2-step cloning process. In the course of this process Atg34-gene constructs with N-terminal fluorescence- and 6xHis-tags were generated. TEV-cleavage sites were introduced via the second PCR.
4.2 SDS-Polyacrylamide Gel-electrophoresis (SDS-PAGE)

Running buffer (1x):
25mM Tris
192mM glycine
0.1% SDS

Polyacrylamide-gels:
Separating gels (12% and 15%)
H$_2$O
Acrylamide/ Bis-acrylamide (30%/ 0.8% w/v)
1.5M Tris (pH=8.8)
10% (w/v) SDS
10% (w/v) ammonium persulfate
TEMED

Stacking gels (5%):
H$_2$O
0.5M Tris-HCl (pH= 6.8)
Acrylamide/ Bis-acrylamide 30%/ 0.8% w/v)
10% (w/v) ammonium persulfate
TEMED

SDS-loading dye (6x):
375mM Tris-HCl (pH 6.8)
6% SDS
48% Glycerol
9% 2-Mercaptoethanol
0.03% Bromphenol Blue

Protein standards:
Precision Plus Protein™ Standard (BioRad)
PageRuler™ Prestained Protein Ladder (Thermo Scientific)

Staining: Coomassie Blue (Brilliant Blue R-250 dye) dissolved in destaining-solution (40% methanol, 10% glacial acetic acid)
Protein samples were mixed with SDS-loading dye (1x) and distilled water was added when necessary, samples were boiled for ~2min in a water-bath. Samples were then loaded onto SDS-acrylamide gels (either 12% or 15% bis-acrylamide). SDS-PAGE was performed at 300V. After protein separation, gels were stained using Coomassie Blue to detect protein bands.

4.3 Protein Expressions and Purifications

Buffers and Solutions:

Lysis-Buffer with Imidazole:
50mM HEPES (pH 7.5)
300mM NaCl
1mM MgCl$_2$
10mM Imidazole
2mM β-mercaptoethanol
Complete Inhibitor (Roche) or pefabloc (Roche)
DNase (Sigma Aldrich)

Lysis Buffer w/o imidazole:
50mM HEPES (pH 7.5)
300mM NaCl
1mM MgCl$_2$
2mM β-mercaptoethanol
Complete Inhibitor (Roche) or pefabloc (Roche)
DNase (Sigma Aldrich)

Ni-NTA-Buffers for affinity-chromatography:
Buffer A:
50mM HEPES (pH 7.5)
300mM NaCl
10mM Imidazole
2mM β-mercaptoethanol
Buffer has to be sterile filtered and degased before use
Buffer B:
50mM HEPES (pH 7.5)
300mM NaCl
300mM Imidazole
2mM β-mercaptoethanol
Buffer has to be sterile filtered and degased before use

Size exclusion chromatography (SEC-) buffer:
25mM HEPES (pH 7.5)
150mM NaCl
1mM DTT
Buffer has to be sterile filtered and degased before use

Wash-Buffer I for glutathione beads-purification:
50mM HEPES (pH 7.5)
300mM NaCl
1mM DTT

Wash-Buffer II for glutathione beads-purification:
50mM HEPES (pH 7.5)
700mM NaCl
1mM DTT

Elution Buffer for glutathione beads-purification:
50mM HEPES (pH 7.5)
300mM NaCl
1mM DTT
10mM reduced glutathione
pH has to be adjusted to 7.5 to 8.0 with HCl

Wash-Buffer for Ni-NTA beads-purification
50mM HEPES (pH 7.5)
300mM NaCl
10mM Imidazole
2mM β-Mercaptoethanol
Ion-Exchange Chromatography Buffer:
1000mM NaCl
50mM HEPES (pH 7.5)
2mM β-Mercaptoethanol

Cell lines:
- *Escherichia coli* BL21 (DE3) pLysS
- *Escherichia coli* Rosetta (DE3) pLysS

Media:
- **LB (Luria Bertani)-Medium:**
  - Peptone 1%
  - Yeast extract 0.5%
  - NaCl 1%

- **TB (Terrific Broth)-Medium:**
  - Peptone 1.2%
  - Yeast extract 2.4%
  - $K_2HPO_4$ 72mM
  - $KH_2PO_4$ 17mM
  - Glycerol 0.4%

Antibiotics (stock solutions):
- Ampicillin 50mg/mL in H$_2$O
- Chloramphenicol 34mg/mL in ethanol
- Kanamycin 50µg/mL in H$_2$O

Enzymes:
- TEV-protease (self-made)
- Thrombin – 1000 units/mg protein (SERVA Electrophoresis)
4.3.1 Test Expressions of mCherry-Atg34- and mEGFP-Atg34-Constructs

4.3.1.1 Transformation of *E. coli* BL21 (DE3) pLysS and *E. coli* Rosetta (DE3) pLysS-Cells

For protein test expression in *E. coli* BL21 (DE3) pLysS and *E. coli* Rosetta (DE3) pLysS cells were transformed with wildtype mCherry-Atg34, mEGFP-Atg34, mCherry-Atg34W409A and mEGFP-Atg34W409A, respectively. Transformation was carried out as described above. Ampicillin (50mg/mL) and chloramphenicol (34mg/mL) were used as selection markers.

4.3.1.2 Inoculation

Three *E. coli* BL21 (DE3) pLysS and *E. coli* Rosetta (DE3) pLysS colonies each containing the above mentioned gene-constructs were picked to inoculate 5mL of LB-medium, using again ampicillin (50mg/mL) and chloramphenicol (34mg/mL) as selection markers. Cultures were grown over night at 37°C at 180rpm.

4.3.1.3 Test Expressions

To define the ideal growing temperature for protein expression and solubility test expressions were carried out at three different growing temperatures. Additionally, different expression times were tried out on order to survey the growing times resulting in the highest protein yield. Test expressions were performed with both *E. coli* strains BL21 (DE3) pLysS and Rosetta (DE3) pLysS containing the wildtype gene-constructs – either mEGFP-Atg34 or mCherry-Atg34 in pET Duet. Temperatures tested were 18°C, 25°C and 37°C, respectively. Samples of the cultures grown at 18°C and 25°C, respectively, were collected after 5 hours and 16 hours (over night). Cultures grown at 37°C were harvested after 3 hours. Ampicillin (50mg/mL) and chloramphenicol (34mg/mL) were used as selection markers. Cultures were grown in 250mL Erlenmeyer flasks containing 100mL of LB-medium each, which were inoculated with 2mL of the pre-cultures (mCherry-Atg34 in *E. coli* Rosetta (DE3) pLysS and *E. coli* BL21 (DE3) pLysS, respectively, mEGFP-Atg34 in *E. coli* Rosetta (DE3) pLysS and *E. coli* BL21 (DE3) pLysS, respectively). Cultures used for test expressions at 18°C and 25°C first were grown at 37°C and 180rpm to an OD$_{600}$ of 0.3 to 0.4. Now the temperature was reduced to 18°C and 25°C, respectively. When an OD$_{600}$ of 0.6 to 1 was reached regardless of the sample
growing conditions, protein-expression was induced by adding Isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.5mM).

4.3.1.4 Sample Collection
For those test expressions carried out at 18°C and 25°C, respectively, samples was taken 6 hours and 16 hours (over night expression) after the induction of protein-expression has taken place. Samples of the test-expressions carried out at 37°C were collected 3 hours after IPTG-treatment. Therefore 4mL of each culture were taken and pelleted by centrifugation at 4000rpm for 5min at 4°C. Cell pellets were resuspended in lysis buffer, flash frozen using liquid N₂ and stored at -80°C.

4.3.1.5 Sample Preparation
Cells were opened by freezing and thawing. Samples of the cell lysates were taken at this point. The rest of the lysates were pelleted by centrifugation at 13,000rpm for 20min at 4°C. Samples of the supernatant were drawn. All samples were prepared for SDS-PAGE and loaded onto 12%-SDS-gels. Gels were stained using Coomassie-Blue.

4.4 Large Scale Protein Expression and Purification of mCherry-ATG34 and mCherry-ATG34W409A
mCherry-tagged Atg34 and Atg34W409A, respectively, in pET Duet-1 were expressed in E. coli Rosetta (DE3) pLysS-cells. Large scale expression was carried out over night at 18°C according to the above mentioned protocol used for the test-expressions. Cells were grown in LB-medium. Ampicillin (50µg/mL) and chloramphenicol (34µg/mL) were used as selection markers. Protein expression was induced using 100µL/L IPTG (1mM). Cells were harvested by centrifugation at 5000rpm and 4°C. The obtained cell pellets were resuspended in lysis buffer, flash frozen using liquid N₂ and stored at -80°C. To exclude the possibility of protein-aggregation samples of the cell lysates and the supernatant obtained after centrifugation were taken and checked by SDS-PAGE on 12%-SDS-gels.

4.4.1 Purification of mCherry-Atg34 and mCherry-Atg34W409A by affinity chromatography
Purification was carried out by affinity chromatography using a Ni²⁺-column as
stationary phase. In theory the 6xhistidine-tag on mCherryAtg34 and mCherryAtg34W409A, respectively would interact with Ni$^{2+}$-ions on the column forming a ionic bond and thus entrapping the protein there, while letting unwanted constituents of the cells supernatant pass. Elution of the protein can then be performed using buffers containing imidazole, which competes with the 6xHis-tag for its binding partners on the column.

4.4.1.1 NiNTA affinity-chromatography

E. coli Rosetta (DE3) pLysS-cells containing mCherry-Atg34 or mCherry-Atg34W409A, respectively, stored at -80°C were opened by thawing followed by one round of sonication. Sonication was carried out for 30s using a MS73/D-tip. To remove insoluble parts of the cells ultracentrifugation was then applied. To this end cells were centrifuged for 45min at 30,000rpm and 4°C. The supernatant was isolated and filtered using a sterile filter (0.45µm pore diameter).

The samples were then loaded onto a 5mL HisTrap-HP column and affinity chromatography was performed. A step gradient of different imidazole concentrations (50 to 300mM imidazole) was used to elute different sample-fractions. 2mL-fractions were collected and analysed using SDS-PAGE to determine in which fractions Atg34 or Atg34W409A, respectively, could be found. Samples of each fraction were therefore mixed with SDS-loading dye (6x) and treated as mentioned above. Fractions containing the protein of interest were pooled and further processed.

4.4.1.2 TEV-cleavage

The protein-solution was concentrated to a total volume of ~2mL by ultrafiltration using Amicon® Ultra-4 Centrifugal Filter Units (Millipore) with a 30kDa or 50kDa molecular weight cut off filter. Centrifugation was carried out at 4000rpm and 4°C. To dilute the imidazole in the sample, SEC-buffer was added to decrease the imidazole concentration to ~30mM. Since a TEV-cleavage site was added via PCR, Tobacco Etch Virus-protease (TEV-protease) was added to cleave off the 6xHis-tag. Incubation was performed for 16h. Successful cleavage of the 6xHis-tag was confirmed by SDS-PAGE on a 12%-SDS-gel.
4.4.1.3 Removal of TEV-Protease
To remove TEV-protease in the protein-sample Ni-NTA magnetic agarose beads (Qiagen) were used. Therefore 1mL of beads were equilibrated using SEC-buffer and added to the protein-sample. Incubation was carried out for 1 hour at 4°C on a shaking platform to bind the remaining TEV-protease to the beads. After incubation beads were collected by centrifugation at 4000rpm for 3min at 4°C.

4.4.1.4 Size Exclusion Chromatography
The protein-samples were filtered using a 45µm-filter to remove any residual Ni-beads and concentrated to a total volume of 2mL using Amicon® Ultra-4 Centrifugal Filter Units (Millipore) with a 50kDa molecular weight cut off filter. The concentrated samples were then loaded onto a Superdex 200 16/60 column and size exclusion chromatography (SEC) was performed. SEC-fractions were collected and checked by SDS-PAGE on 12% SDS-gels. Those fractions containing mCherryAtg34 and mCherryAtg34W409A, respectively, were pooled.

4.4.1.5 Measurement of Protein-Concentrations
Protein-concentrations were measured photometrically at an absorption wavelength of 280nm. SEC-buffer was used as a reference and for dilutions. Protein-concentrations were calculated according to the following formula: 

\[ c (M) = \frac{A_{280nm}}{\varepsilon} \]

In case of low protein-concentrations, protein-solutions were concentrated again (Amicon® Ultra-4 Centrifugal Filter Units (Millipore) with a 50kDa molecular weight cut off filter). Then aliquots between 15 and 100µL were prepared, flash-frozen using liquid N\(_2\) and stored at -80°C.

4.4.2 Large Scale Protein Expression and Purification of GST-Atg34 and GST-Atg34W409A
A version of both Atg34 and mutant Atg34W409A respectively, both N-terminally tagged with GST were produced for GST-Pull Downs. Therefore \textit{E. coli} Rosetta (DE3) pLysS-cells were transformed with pGEX4TI containing these proteins. Transformation was carried out according to the already mentioned protocol. 40mL of the over night cultures of the transformed \textit{E. coli} Rosetta (DE3) pLysS-cells were then taken to inoculate 1L of LB-medium. Chloramphenicol (34µg/mL)
and Ampicillin (50µg/mL) were used as selection markers. Cultures were grown at 37°C and 180rpm until an OD<sub>600</sub> of ~0.3 was reached. Then incubation-temperature was decreased to 18°C. Protein-expression was induced at an OD<sub>600</sub> of ~0.6 by adding IPTG (1mM). Additionally, rotation speed was decreased to 140rpm and cultures were grown over night. Afterward cells were harvested by centrifugation at 5000rpm and 4°C for 10min and resulting cell pellets were resuspended in lysis-buffer w/o imidazole on a shaking platform at 4°C. Dissolved cell-pellets were then flash-frozen using liquid N<sub>2</sub> and stored at -80°C.

4.4.2.1 Purification of GST-Atg34 and GST-Atg34W409 using Glutathione sepharose 4B beads

For the purification of GST-Atg34 and GST-Atg34W409A Glutathione sepharose 4B beads (GE-Healthcare) were used. Therefore these beads were equilibrated before with wash-buffer 1 by two rounds of centrifugation at 4000rpm, 4°C for 2min each.

E. coli Rosetta (DE3) pLysS-cells containing GST-Atg34 or GST-Atg34W409A, respectively, were lysed by gentle thawing in a water-bath followed by 30s of sonication (tip 73D). Ultracentrifugation at 40.000rpm and 4°C for 45min was used to remove insoluble material. The remaining supernatant was then loaded onto the equilibrated GST-beads and incubated for 2h at 4°C on a rotating platform in order to bind the protein to the resin. After incubation washing steps were performed using Wash Buffer I and Wash Buffer II as described below. All incubation steps were carried out at 4°C on a rotating platform, all centrifugation steps were carried out at 4000rpm and 4°C for 2min. Washing consisted of 5 rounds of 5min washing with Wash-Buffer I followed by 1 round of 30min washing using Wash-Buffer II and finally with 2 rounds of 5min washes using again Wash-Buffer I. Elution of the purified protein was achieved by adding an equal volume of Elution Buffer containing glutathione (10mM) to the beads. After 30min of incubation at 4°C on a rotating platform the suspension was centrifuged at 4000rpm and 4°C for 2min.

For later size exclusion chromatography the eluate was filtered using a 45µm-filter and concentrated to a total volume of 2mL using Amicon® Ultra-4 Centrifugal Filter Units (Millipore) with a 50kDa molecular weight cut off filter. Successful protein elution was additionally controlled by SDS-PAGE on 12% SDS-gels.
4.4.2.2 Size Exclusion Chromatography
The sample was then loaded onto a Superdex 200 16/60-column and size exclusion chromatography was performed. Peak fractions were selected and on a SDS-PAGE. Fractions containing GST-Atg34 or GST-Atg34W409A, respectively, were pooled and concentrated using Amicon® Ultra-4 Centrifugal Filter Units (Millipore) with a 30kDa molecular weight cut off filter to a total volume of 1mL. Protein-concentration was measured photometrically at 280nm. Samples were then flash-frozen in liquid N₂ and stored at -80°C.

4.4.2.3 Thrombin-Cleavage
For one part of the GUVs-assays untagged Atg34 and Atg34W409A, respectively, were needed, thus a thrombin-cleavage was performed after beads-purification with Glutathione Sepharose 4B-beads (GE-Healthcare). Therefore after the final washing step thrombin (SERVA Electrophoresis) was used to cleave off the protein from the GST-tag. Incubation was performed over night at 4°C at a rotating platform. Successful cleavage was confirmed by SDS-PAGE on 12%-SDS-gels.

4.4.3 Large Scale Protein Expression and Purification of Atg8
Atg8 containing a 6xHis-tag at its N-terminus was expressed. Therefore E. coli Rosetta (DE3) pLysS-cells containing pET-Duet with 6xHis-Atg8 from a glycerol stock were used to inoculate LB-medium. Ampicillin (50µg/mL) and chloramphenicol (34µg/mL) were used as selection markers. The culture was grown over night at 37°C and 220rpm.

With this over night culture TB-medium was inoculated. Ampicillin (50µg/mL) and chloramphenicol (34µg/mL) were used as selection markers. The culture was grown at 37°C and 180rpm to an OD₆₀₀ of ~0.6; then protein-expression was induced by adding IPTG (0.5 mM). 3h later cells were harvested by centrifugation at 5000rpm and 4°C for 10min. Resulting cell-pellets were dissolved in lysis-buffer containing imidazole and flash-frozen in liquid N₂. Samples were stored at -80°C.

4.4.3.1 Purification of Atg8
Cells were lysed by gentle thawing in a water-bath and 30s of sonication (tip 73D). Then Ultracentrifugation was performed at 40,000rpm and 4°C for 40min to remove insoluble material. The soluble fraction was then filtered through 0.45µm-
filter units (millipore) and loaded onto a HisTRAP 5mL HP-column (GE Healthcare) to perform affinity-chromatography as described before. Fractions eluted at an imidazole-concentration of 150mM were collected, pooled and concentrated using Amicon® Ultra-4 Centrifugal Filter Units (Millipore) with a 30kDa molecular weight cut off filter to a total volume of 3mL.

**TEV-cleavage:**
To remove the 6xHis-tag on Atg8 the 3mL protein-solution was diluted 10x with SEC-buffer and 1mL of TEV-protease was added. Incubation was done at room temperature on a shaking platform for 13h. Successful TEV-cleavage was confirmed by SDS-PAGE on a 15% SDS-gel.

### 4.4.3.2 6xHis-tag protein-purification with Ni-NTA beads
To remove the remaining active TEV-protease in the protein-sample Ni-NTA agarose beads (Qiagen) were used. Therefore beads were equilibrated using SEC-buffer and loaded onto the protein-sample. Incubation was carried out for 2h at 4°C on a shaking platform to bind the remaining TEV-protease to the beads. After incubation beads were collected by centrifugation at 4000rpm and 4°C for 10min. For later size exclusion chromatography the supernatant was filtered (Millipore, 45µm pore diameter) and concentrated to a total volume of 2mL using Amicon® Ultra-4 Centrifugal Filter Units (Millipore) with a 30kDa molecular weight cut off filter.

### 4.4.3.3 Size Exclusion Chromatography of Atg8
The sample was loaded onto a S75 16/60 column and SEC was performed. Peak fractions were collected and protein content was checked with SDS-PAGE on a 15%-SDS gel. Those fractions containing the protein were pooled and concentrated again using Amicon® Ultra-4 Centrifugal Filter Units (Millipore) with a 30kDa molecular weight cut off filter to a total volume of ~1mL. Then protein-concentration was photometrically measured at OD_{280}. Samples were flash-frozen in N₂ and stored at -80°C.

### 4.4.4 Large Scale Expression and Purification of Atg7
Atg7 – as mentioned before – is a part of the conjugation-complex that binds Atg8 to the phagophore and was used for the later described GUV-assays.
4.4.4.1 Transformation
For transformation the plasmid pOPTH carrying the Atg7-insert containing a N-terminal 6xHis-tag was used to transform *E. coli* BL21 (DE3) pLysS-cells. Transformation was done by heat shock as described in the before mentioned transformation-protocol.

4.4.4.2 Large Scale Protein Expression of Atg7
Resulting colonies from the transformation were picked and over night cultures were prepared using LB-medium and Ampicillin (50µg/mL) and Chloramphenicol (34µg/mL) as selection markers. These cultures were then used to inoculate TB-medium containing Ampicillin and Chloramphenicol. These cultures were grown at 37°C and 180rpm until an OD$_{600}$ of ~0.3 was reached, then incubation temperature was reduced to 18°C. Induction of protein expression took place at an OD$_{600}$ of 0.7 by adding IPTG (0.5mM). Rotating speed was reduced to 140rpm and cultures were grown over night.
Cells were harvested as described above.

4.4.4.3 Purification of Atg7
Cells were lysed by gentle thawing in a water-bath and 30s of sonication (tip 73D). Then Ultracentrifugation was performed at 40.000rpm and 4°C for 40min to remove insoluble material.

Purification with Ni-NTA agarose beads: The supernatant resulting from the ultracentrifugation was loaded onto Ni-NTA magnetic agarose beads (Qiagen). The beads therefore were pre-equilibrated with washing-buffer containing imidazole (10mM).

An equal volume of beads and supernatant were then incubated together for 2h at 4°C to bind Atg7 via its 6xHis-tag to the resin. Then beads were washed with washing-buffer as described below. All centrifugation steps in between were carried out at 4000rpm and 4°C for 2min. Washing consisted of 4 rounds of a 5min-wash followed by 1 round of a 30min-wash and ending with 2 rounds of 5min-washing. Finally the beads with bound Atg7 were resuspended in an equal volume of washing-buffer and TEV-protease was added.
4.4.4.4 TEV-cleavage

1mL of TEV-protease was added to the suspension in order to cleave off the 6xHis-tag for further purification. Incubation was carried out over night at room temperature on a shaking platform. The suspension was then centrifuged at 4000rpm and 4°C for 2min to sediment the beads in order to remove them from the cleaved and thus soluble Atg7 in the supernatant. Beads were then washed 2 times with washing-buffer, the supernatant was put aside and the protein-content was checked using SDS-PAGE on a 12%-SDS gel. Both the supernatant samples were then pooled.

4.4.4.5 Ion-Exchange Chromatography of Atg7

In ion exchange chromatography analytes are retained at the column via ionic interactions of ionic residues of the stationary phase on the column and ionic residues of the analyte. Elution is then achieved by washing the column with gradually increasing amounts of similarly charged ions in the mobile phase. These similarly charged components compete with the functional groups of the analyte for their binding partners on the column.

In the case of Atg7 cation exchange chromatography was performed. Elution was carried out with a buffer containing Na+-ions to compete for the analytes binding partners on the column.

In order to reduce the high salt-concentration in the protein-solution, which would interfere with the binding of the Atg7 to the column, it was first diluted 1:2 with HEPES (50mM, pH 7.5) to a final concentration of ~150mM. Then the sample was concentrated to a total volume of 6mL using Amicon® Ultra-4 Centrifugal Filter Units (Millipore) with a 50kDa molecular weight cut off filter. The solution was then loaded onto a 16/60 Q-Sepharose-column (Superdex) and ion-exchange chromatography was performed with an ion-gradient of 150 to 1000mmol NaCl.

Peak fractions were then run on a 12%-SDS gel by SDS-PAGE to analyze which fractions contained Atg7. Fractions containing pure Atg7 were pooled and concentrated using an Amicon® Ultra-4 Centrifugal Filter Unit (Millipore) with a 50kDa molecular weight cut off filter to a total volume of 2mL.

4.4.4.6 Size Exclusion Chromatography of Atg7

The concentrated protein-sample was filtered (Millipore, 45μm pore diameter) and
loaded onto a 16/60 Superdex 200 column to perform SEC. Peak fractions were then pooled and concentrated to a total volume of 220µL using Amicon® Ultra-4 Centrifugal Filter Units (Millipore) with a 30kDa molecular weight cut off filter. The protein-concentration was then determined photometrically at 280nm. Finally a glycerol stock of Atg7 was prepared in a 1:1 dilution with glycerol (85%) and stored at -20°C.

### 4.5 Protein-Interaction Assays with Giant Unilamellar Vesicles

Giant Unilamellar Vesicles (GUVs) are artificially generated membrane-like amphiphile structures with a size of ~1 – 50µm in diameter that surround an aqueous phase. The single membrane is composed of phospholipids of arbitrary composition. GUVs can easily be observed with light-microscopy and in addition fluorescent-dye can be incorporated into their membranes. Different methods exist to generate GUV. The most widely used method is the hydration of a thin lipid film positioned on a solid surface in combination with an applied electric field. This method is referred to as electroformation [14].

Thus to generate GUVs a lipid-mixture (1µM, 3µL per glass slide) was applied to a conductive glass plate coated with indium tin oxide (ITO) in small droplets and dried in an exicator for up to 4h. An aqueous sucrose-solution (5%) was added for hydration purpose and an electric field was applied using the following protocol:

- Phase I: 30min with a sine wave frequency of 10Hz and a peak to peak-amplitude ramped up linearly from 0.05V to 1.41V.
- Phase II: 120min with a sine wave frequency of 10Hz and a constant peak to peak-amplitude of 1.41V
- Phase III: 30min with a sine wave frequency of 4.5Hz and a constant peak to peak-amplitude of 2.12V

GUVs in sucrose-solution were then transferred into an Eppendorf-tube for further proceeding. These artificially produced GUVs were used to analyze the Atg8-dependent recruitment of Atg34 to the lipid membrane. To observe this process fluorescence microscopy was conducted and different experimental set ups were implemented. In the first set up a mEGFP-tagged version of Atg8 was used to analyze the recruitment of mCherry-Atg34 and mCherry-Atg34W409A, respectively, to the GUVs, which were fluorescently-labeled with a Marina Blue-
modified lipid. The same recruitment was analyzed using a His-tagged version of mEGFP-tagged Atg8 and GUVs containing Ni-NTA-lipids in another set up. To analyze the cargo-recruitment to the membrane UltraClean™ high activity latex beads (Invitrogen) were coated with fluorescence-labeled Atg34 and Atg34W409A, respectively, and the interaction with Atg8 on the GUVs was studied. Wildfield microscopy images were acquired using Olympus Cell^R imaging station with x 60 or x 100 oil objectives (NA 1.4). Image processing and analyzing was carried out using ImageJ software.

Lipid-mixtures for GUVs:

Lipid-mixture I:
38% DOPC (Avanti)
35% DOPS (Avanti)
20% DOPE (Avanti)
5% PI(3)P (Avanti)
2% Marina Blue coupled to PE (Avanti)

Lipid-mixture II:
12% DOPC (Avanti)
3% DOPE (Avanti)
2% DOPS (Avanti)
26% DGS-NTA(Ni) (Avanti)
44% DPPC (Avanti)
13% Cholesterol (Avanti)

Lipid-mixture III:
90% POPC (Avanti)
10% DGS-NTA(Ni) (Avanti)

Buffers and Solutions:
Tris Buffered Saline (TBS):
50mM Tris-Cl (pH 7.5)
150mM NaCl
Bovine Serum Albumin (BSA) Solution for coating of microscopy dishes:
2.5mg/mL in 1x Tris Buffered Saline (TBS)

BSA-solution for UltraClean™ high activity latex beads:
5mg/mL BSA in
135mM NaCl
15mM HEPES (pH 7.5)

GUV-buffer:
135mM NaCl
15mM HEPES (pH 7.5)

2-(N-morpholino)ethanesulfonic acid (MES-buffer) (pH 6.0)
Phosphate Buffered Saline (PBS) - 50mM (pH 7.4) plus 0.9% NaCl

Glycine – 2M, dissolved in H₂O

Protein-Crosslinker:
1-Ethyl-3-(3-dimethylaminopropyl)carbodiimid (EDAC) (Uptima) - 80mg/mL in H₂O

Enzymes:
Atg8-Mix:
mEGFP-Atg8 (0.4µM)
Atg7 (0.08µM)
Atg3 (0.08µM)
MgCl₂ (0.5mM)
ATP (1mM)
dissolved in GUV-buffer
6xHis-tagged mEGFP-Atg8, 4µM
Conjugation-complex – Atg12-Atg5-Atg16, 4µM

4.5.1 GUV-assays with Conjugation System
For the preparation of GUVs lipid mixture I was used, composed of the following lipids (Avanti) were 1,2-dioleoyl-sn-glycer-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS), 1,2-dioleoyl-sn-glycero-3-
phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-3'-phosphate) (PI(3)P) and Marina Blue fluorescence dye coupled to phosphoethanolamine were used. The lipid-mixture was concentrated to 2/3 of its volume using argon gas and diluted 2:1 with methanol resulting in a concentration of 10mg/mL.

GUVs were prepared as mentioned above. Multi-well glass bottom microscopy-dishes were coated with Bovine Serum Albumin (BSA, 2.5mg/mL in 1x TBS) for 1h at room temperature. BSA was then removed and the plates were washed with buffer. GUVs in sucrose-solution (30µL), Atg12-Atg5-Atg16-conjugation complex (0.4µM, 4µL) and mEGFP-Atg8-mix (4µL) were added to wells and incubated for 40min in the dark to facilitate the conjugation of Atg8 to DOPE in the GUV-membranes.

Then mCherry-Atg34 or mCherry-Atg34W409A, respectively, were added and incubated again for 30min. Results were studied using fluorescence microscopy (Olympus Cell-R). Wells containing only the conjugation-complex and mEGFP-Atg8-mix, as well as wells containing only mCherry-Atg34 and mCherry-Atg34W409A were used as controls.

4.5.2 GUV-assay with DGS-NTA(Ni)-lipids (phase separated GUVs)

For the preparation of GUVs the following lipids (Avanti) were used 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-[N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt) (DGS-NTA(Ni)), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and Cholesterol were used. The lipid-mixture was concentrated to 2/3 of its volume using argon gas and diluted 2:1 with methanol.

Electroformation of the lipid-mixture was carried out at 55°C for 3h. Multi-well microscopy dishes were prepared with BSA as described before. 6xHis-tagged mEGFP-Atg8 was used, that bound directly to the Ni-residues of the GUVs via its 6xHis-tag. GUVs were incubated with mEGFP-Atg8 (0.4µM) for 10min, before mCherry-Atg34 (0.4µM) and mCherry-Atg34W409A (0.4µM), respectively, were added as well. Results were studied using fluorescence microscopy (Olympus Cell-R). Wells containing only mEGFP-Atg8, as well as wells containing only mCherry-Atg34 and mCherry-Ag34W409A were used as controls.
4.5.3 GUV-assay with POPC and DGS-NTA(Ni)-lipids

For the preparation of these GUVs lipid mixture II was used, containing only 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt) (DGS-NTA(Ni)). The lipid-mixture was concentrated to 2/3 of its volume using argon gas and diluted 2:1 with methanol. Electroformation was carried out as described before. Multi-well microscopy dishes were coated with BSA as described before. 6xHis-tagged mEGFP-Atg8 (4µM) was used, that bound directly to the Ni-residues of the GUVs via its 6xHis-tag. GUVs were incubated with mEGFP-Atg8 for 10min, before mCherry-Atg34 (0.4µM) and mCherry-Atg34W409A (0.4µM), respectively, were added as well. Results were studied using fluorescence microscopy (Olympus Cell-R). Wells containing only mEGFP-Atg8, as well as wells containing only mCherry-Atg34 and mCherry-Atg34W409A were used as controls.

4.6 GST-Pulldown Assay

To detect the interaction of Atg8 with Atg34 in solution GST-pulldown assays were conducted according to those described by Noda et al. [15] but using the full-length protein instead of only its C-terminus. Glutathione sepharose 4B-beads (GE Healthcare) were used. GST binds strongly to Glutathione, thus a protein tagged with GST binds to the beads. If this protein now binds to another protein, both would be present on the beads and can therefor be pelleted and subsequently detected via SDS-PAGE. Therefor this assay is a valuable tool to determine protein-protein-interactions.

GST-Pulldown-Buffer:
25mM HEPES (pH 7.5)
150mM NaCl
1mM DTT

Proteins:
GST-Atg34
GST-Atg34W409A
Atg8
GST
For this assay N-terminally GST-tagged versions of Atg34 and Atg34W409A, respectively, and an untagged version of Atg8 was used. Beads (15µL) with a binding capacity of 5mg/mL were pre-equilibrated with buffer. Then the beads were incubated with GST-Atg34 (12.5µM) and GST-Atg34W409A (12.5µM), respectively, together with untagged Atg8 (20µM) on a turning wheel at 4°C. After 1h of incubation, the mix was centrifuged at 4000rpm at 4°C for 4min and the unbound prey (Atg8) was collected. This was followed by three 5min washing steps. At the end the beads were resuspended in sample-buffer, prepared for SDS-PAGE and results were checked on 15% SDS-gels. Incubation of beads with GST and Atg8 (20µM each) and Atg8 (20µM) alone were used as controls.

4.7 Complexation-Formation Experiment by Using Analytical Size Exclusion Chromatography

SEC-Buffer w/o DTT:
25mM HEPES (pH 7.5)
150mM NaCl

To gain information about the concentration dependence of the Atg8-Atg34-interaction a complex-formation experiment was conducted using analytical size exclusion chromatography (SEC). Atg34 and Atg34W409A were freshly used with no freezing and thawing cycles in between purification and the experiment. Atg8 was purified in advance and stored at -80°C. Protein samples were concentrated using Amicon® Ultra-4 Centrifugal Filter Units (Millipore) with a 50kDa molecular weight cut off filter. Concentrations reached from 25µM to 1960µM – these fractions were loaded onto a Superose 6 3.2/30 column and analytical SEC was performed. Atg34 and Atg34W409A-interaction with Atg8 was studied under low (40µM each) and high (740µM) protein concentrations. Therefore samples of Atg34 and Atg8 and Atg34W409A and Atg8, respectively, were loaded onto a Superose 6 3.2/30 column and analytical SEC was performed. As controls Atg34, Atg34W409A and Atg8 were run alone as well. After every run, fractions were analyzed by SDS-PAGE on 15% SDS-gels.
5. Results

5.1 6xHis-tagged mCherry-Atg34-, mCherry-Atg34W409A-, mEGFP-Atg34- and mEGFP-Atg34W409A-constructs were designed

For later protein interaction experiments on the membrane - using GUVs - 6xHis-tagged mCherry-Atg34- and mEGFP-Atg34-constructs were designed. Simultaneously mutant versions of these proteins were constructed, where tryptophane was exchanged by alanine in the AIM-sequence. The mutant versions were used as negative controls in the course of the following experiments. To obtain these constructs a two-step cloning process was used to first introduce a fluorescence-tag (mCherry or mEGFP (monomeric GFP, enhanced due to an A206K-mutation [38])) N-terminally to both the wildtype and the mutant protein-sequence of Atg34, followed by the introduction of a N-terminal 6xHis-tag to all protein-constructs. A TEV-cleavage site was added via the second PCR step to be able to cleave off the 6xHis-Tag during the following protein purification procedure. The cloning process resulted in the constructs shown in figure 13. Successful cloning was confirmed by sequencing (LGC Genomics, Sanger sequencing). See section 4.1 for detailed procedure description.

Fig. 13: Atg34 and Atg34W409A gene-constructs derived from the two-step cloning process. Constructs were designed comprising either a N-terminal mCherry or mEGFP fluorescence-tag, followed by a Histidine-tag and a TEV-cleavage site in between both tags.
5.2 Test-expressions of Atg34-fusion proteins indicate ideal expression conditions for mCherry-Atg34 and mEGFP-Atg34

mCherry-Atg34 and mEGFP-Atg34 were expressed in *E. coli* BL21 (DE3) pLysS- and Rosetta (DE3) pLysS-cells under different growing conditions (18°C for 6h and over night, 25°C for 6h and over night, and 37°C for 3h after IPTG addition (1mM)). Samples were taken at the point of cell-harvesting. Samples were lysed by freezing and thawing and centrifuged. Lysates and soluble fractions were processed and loaded onto 12%-SDS gels as described in sections 4.2 and 4.3. 6xHis-mCherry-Atg34 has a molecular weight of 79kDa and 6xHis-mEGFP-Atg34 a molecular weight of 77kDa. Thus the corresponding bands appear slightly above the height of the 75kDa band of the used protein standard (Precision Plus Protein Standard (BioRad), depicted in figure 14). As shown in figures 15 - 19 the most prominent bands compared to the uninduced control where observed for *E. coli* Rosetta pLysS-cells when grown at 18°C over night for mCherry-Atg34 and for mEGFP-Atg34, respectively when grown at 25°C for 5h.

![Fig. 14: Precision Plus Protein Standard (BioRad).](image)
Fig. 15: Test Expression of mCherry-Atg34 and mEGFP-Atg34 at 25°C and 5h. Loading Scheme: 1 and 2: Uninduced control (mCherry-Atg34W409A in *E. coli* Rosetta (DE3) pLysS); 3 and 4: mCherry-Atg34 in *E. coli* Rosetta (DE3) pLysS; 5 and 6: mCherry-Atg34 in *E. coli* BL21 (DE3) pLysS; 7 and 8: mEGFP-Atg34 in *E. coli* Rosetta (DE3) pLysS; 9 and 10: mEGFP-Atg34 in *E. coli* BL21 (DE3) pLysS.
Fig. 16: Test Expression of mCherry-Atg34 and mEGFP-Atg34 at 25°C over night. Loading Scheme: 1 and 2: Uninduced control (mCherry-Atg34W409A in *E. coli* Rosetta (DE3) pLysS); 3 and 4: mCherry-Atg34 in *E. coli* Rosetta (DE3) pLysS; 5 and 6: mCherry-Atg34 in *E. coli* BL21 (DE3) pLysS; 7 and 8: mEGFP-Atg34 in *E. coli* Rosetta (DE3) pLysS; 9 and 10: mEGFP-Atg34 in *E. coli* BL21 (DE3) pLysS.
Fig. 17: Test Expression of mCherry-Atg34 and mEGFP-Atg34 at 18°C and 5h. Loading Scheme: 1 and 2: Uninduced control (mCherry-Atg34W409A in *E. coli* Rosetta (DE3) pLysS); 3 and 4: mCherry-Atg34 in *E. coli* Rosetta (DE3) pLysS; 5 and 6: mCherry-Atg34 in *E. coli* BL21 (DE3) pLysS; 7 and 8: mEGFP-Atg34 in *E. coli* Rosetta (DE3) pLysS; 9 and 10: mEGFP-Atg34 in *E. coli* BL21 (DE3) pLysS.
Fig. 18: Test Expression of mCherry-Atg34 and mEGFP-Atg34 at 18°C over night. Loading Scheme: 1 and 2: Uninduced control (mCherry-Atg34W409A in *E. coli* Rosetta (DE3) pLysS); 3 and 4: mCherry-Atg34 in *E. coli* Rosetta (DE3) pLysS; 5 and 6: mCherry-Atg34 in *E. coli* BL21 (DE3) pLysS; 7 and 8: mEGFP-Atg34 in *E. coli* Rosetta (DE3) pLysS; 9 and 10: mEGFP-Atg34 in *E. coli* BL21 (DE3) pLysS
5.3 Protein Expressions and Purifications

5.3.1 Large Scale Protein Expression of 6xHis-tagged mCherry-Atg34 and mCherry-Atg34W409A

Both proteins were expressed at 18°C over night and purified using affinity-chromatography and size-exclusion chromatography as described in section 4.3 (figure 20).

Affinity-chromatography: Samples of the flow through and of each peak fraction corresponding to different imidazole concentrations (50mM, 75mM, 100mM, 150mM, 200mM and 300mM) were taken and loaded onto 12%-SDS gels. As shown in figure 21 the protein eluted at 150mM imidazole showed the highest
protein yield. Thus all fractions eluted at 150mM imidazole concentrations were pooled and TEV-protease was added to cleave off the 6xHis-tag. Cleavage was confirmed by SDS-PAGE on 12% SDS-gels as can be seen in figure 22. After removal of the TEV-protease, the samples were concentrated and SEC (Superdex 200 16/60 column) was performed. Samples of the peak fractions were collected, processed and loaded onto 12%-SDS gels. The fractions eluted at ~60mL contained mCherry-Atg34, or mCherry-Atg34W409A, respectively (figures 23 and 24). After pooling of the corresponding fractions and concentrating, protein concentration was determined to be 122µM for mCherry-Atg34 and 840µM for mCherry-Atg34W409A – in a total yield of 1200µL of mCherry-Atg34 and 500µL of mCherry-Atg34W409A.
Fig. 20: Affinity-chromatography of mCherryAtg34. Peak fractions were taken and loaded onto a 12% SDS-Gel.
Fig. 21: 12% SDS-gel of peak fractions obtained from affinity chromatography of mCherryAtg34. After SDS-PAGE and Coomassie Blue staining, it was observed that the 150mM Imidazole fraction contained the mCherryAtg34 protein. These fractions were pooled and purification was continued.
Fig. 22: TEV cleavage of mCherryAtg34. After NiNTA-purification the 6xHis-tag was removed by addition of TEV-protease. Cleavage was checked on 12% SDS-gels using SDS-PAGE and Coomassie Blue staining.
Fig. 23: Size exclusion chromatography of mCherryAtg34.
Fig. 24: SDS-PAGE of peak fraction samples obtained from SEC. After SEC samples of the peak fractions were taken and protein content was determined by SDS-PAGE and Coomassie Blue staining. Fractions 26 to 31 were loaded onto the gel. Pooled fractions are indicated in the figure.

5.3.2 Large Scale Protein Expression and Purification of GST-Atg34 and GST-Atg34W409

Both proteins were expressed at 18°C over night in E. coli Rosetta (DE3) pLysS cells in a volume of 4L and purified using affinity-chromatography with glutathione sepharose 4B-beads (GE Healthcare) followed by size-exclusion chromatography (SEC) as described before (section 4.3). Beads-purification was checked on 12%SDS gels, results are shown in figure 25 and 28. After removal of the beads, samples were concentrated and SEC (Superdex 200 16/60 column) was performed. Samples of the peak fractions were collected, processed and loaded onto 12%-SDS gels. The fractions that eluted around 60mL contained GST-Atg34, or GST-Atg34W409A, respectively. These findings are shown in figures 26 - 30. The corresponding fractions were pooled and concentrated. The protein concentration was determined to be 5.8µM for GST-Atg34 (total yield of 430µL) and 12.72µM (total yield of 530µL) for mCherry-Atg34W409A.
Fig. 25: Purification of GST-Atg34 using glutathione sepharose 4B-beads (GE Healthcare). These beads were used to purify GST-Atg34 from the soluble fraction after protein expression. The eluted fraction containing GST-Atg34 was then loaded onto an Superdex S200 16/60 column and SEC was conducted.
Fig. 26: SEC-purification of GST-Atg34. After purification of GST-Atg34 using glutathione sepharose 4B-beads (GE Healthcare) the eluted fraction containing GST-Atg34 was loaded onto an Superdex S200 16/60 column and SEC was conducted.
Fig. 27: SDS-PAGE of GST-Atg34 of the peak fractions obtained from SEC. Samples of the peak fractions (fractions 26 to 32) were separated on a 12% SDS-gel and stained using Coomassie Blue. Fractions containing GST-Atg34 were then pooled and concentrated.
Fig. 28: Purification of GST-Atg34W409A using Glutathione Sepharose 4B-beads. The beads (GE Healthcare) were used to purify GST-Atg34W409A from the soluble fraction after protein expression.
Fig. 29: Size exclusion chromatography of GST-ATG34W409A purification. The eluted fraction resulting from the Glutathione Sepahrose 4B-beads-purification containing GST-Atg34W09A was then loaded onto an Superdex S200 16/60 column and SEC was conducted.
Fig. 30: SDS-PAGE of GST-Atg34W409A of the peak fractions obtained from SEC. Samples of the peak fractions were then separated on a 12% SDS-gel and stained using Coomassie Blue. Fractions containing GST-Atg34W409A were then pooled and concentrated.

5.3.2.1 Large Scale Protein Expression and Purification of GST-Atg34 and GST-Atg34W409 and Cleavage of the GST-Tag

Both proteins were expressed at 18°C over night in E. coli Rosetta (DE3) pLysS cells in a volume of 4L each and purified using affinity-chromatography with Glutathione Sepahrose 4B-beads (GE Healthcare) and size-exclusion chromatography (SEC) as described before.

After the affinity purification thrombin was added to cleave off the GST-tag. Successful purification and thrombin cleavage was checked on 12%SDS gels. The results are shown in figures 31 - 33.

After removal of the beads, samples were concentrated and SEC was performed. Samples of the peak fractions were collected, processed and loaded onto 12%-SDS gels. The fractions eluted around 60mL contained Atg34, or Atg34W409A, respectively. After pooling of the corresponding fractions and concentrating, protein
concentration was determined to be 23µM for Atg34 and 7.4µM for Atg34W409A.

![Fig. 31: Purification of GST-Atg34 using Glutathione Sepahrose 4B-beads. Glutathione Sepahrose 4B-beads (GE Healthcare) were used to purify GST-Atg34 from the soluble fraction after protein expression. The GST-tag was then cleaved off by Thrombin.](image-url)
Fig. 32: Size exclusion chromatogram of GST-ATG34 purification. After removal of the beads, free Atg34 was loaded onto an Superdex S200 16/60 column and SEC was conducted.
5.3.3 Large Scale Expression and Purification of 6xHis-Atg8

6xHis-tagged Atg8 was expressed at 37°C for 3h and purified using affinity-chromatography followed by size-exclusion chromatography as described in section 4.2.

Affinity-chromatography samples of the flow through and of each peak fraction were taken corresponding to different imidazole concentrations (50mM, 75mM, 100mM, 150mM, 200mM and 300mM) of the eluent and loaded onto 15%-SDS gels. The sample eluted at 150mM imidazole showed the highest Atg8 yield. Thus all fractions 150mM imidazole fractions were pooled and TEV-protease was added to cleave off the 6xHis-tag. Cleavage was confirmed by SDS-PAGE on 15% SDS-gels. 6xHis-Atg8 purification by NiNTA-affinity-chromatography followed by TEV-protease cleavage is shown in figures 34 - 36.

After removal of TEV-protease using Ni²⁺-beads, the samples were concentrated and SEC (Superdex S75 16/60 column) was performed. Samples of the peak fractions were processed and loaded onto 15%-SDS gels. The fractions eluted
around 75mL contained Atg8. SEC chromatographs and SDS-PAGE of corresponding peak fractions are shown in figures 37 - 38. After pooling of the corresponding fractions and concentrating, the protein concentration was determined to be 1.298 mM in a total yield of 980 µL.
Fig. 34: 6xHis-tagged Atg8 purification by NiNTA-affinity-chromatography. Affinity-chromatography using an imidazole gradient (50 to 300mM imidazole), 6xHis-Atg8 eluted at 150mM imidazole (figure 35).
Fig. 35: SDS-gel of peak fractions obtained from affinity chromatography of 6xHis-Atg8. Cell lysate, Input (soluble fraction), flow through and fractions eluted at different imidazole concentrations were loaded onto 15%-SDS gels, after SDS-PAGE 6xHis-tagged Atg8 was detected by coomassie blue-staining.
Fig. 36: TEV-cleavage of 6xHis-Atg8. 6xHis-tags were cleaved of by TEV-protease. Cleavage was checked on 15%-SDS gels by SDS-PAGE and coomassie blue-staining.
Fig. 37: Size exclusion chromatography of ATG8 purification. After 6xHis-tag removal free, concentrated Atg8 was loaded onto an Superdex S75 16/60 column and SEC was conducted.
5.3.4 Large Scale Expression and Purification of 6xHis-tagged Atg7

6xHis-tagged Atg7 was expressed at 18°C over night in *E. coli* BL21 (DE3) pLysS-cells and purified using NiNTA-beads purification, ion-exchange chromatography and size-exclusion chromatography as described in section 4.2. TEV-protease was added after NiNTA-beads purification to cleave off the 6xHis-tag. Successful NiNTA-beads purification and TEV-cleavage was checked on a 12%-SDS gel. As shown in figure 39, not all of the protein was successfully cleaved by TEV and a considerably amount of protein remained on the beads even after a repeating the procedure a second time. The remaining supernatant was purified by ion-exchange chromatography, peak fractions were collected and checked on a 12%-SDS gel. Fractions containing Atg7 were pooled, concentrated and SEC (Superdex 200 16/60 column) was performed. Peak fractions of SEC were again collected and loaded onto a 12%-SDS gel. Fractions containing the desired protein were collected, concentrated and protein-concentration was determined photometrically to be 23.4μM in a total
yield of 220µL. Atg7 purification steps are depicted in figures 39 – 43.

Fig. 39: Purification of 6xHis-Atg7 using NiNTA-beads. Successful NiNTA-beads purification and TEV-cleavage was checked on a 12%-SDS gel with Coomassie Blue-staining.
Fig. 40: Ion-Exchange Chromatography of Atg7.
Fig. 41: SDS-PAGE after Ion-Exchange Chromatography of Atg7. Fractions 32 to 37 were loaded onto a 12%-SDS gel. Fractions containing Atg7 were pooled and processed for SEC.
Fig. 42: Size exclusion chromatography of ATG7 purification. Concentrated Atg7 was loaded onto a Superdex 200 16/60 column and SEC was conducted.
5.4 Protein-Interaction Assays with Giant Unilamellar Vesicles

5.4.1 GUV-assays with mEGFP-Atg8, mCherry-Atg34 and mCherry-Atg34W409A

In the presence of Atg12-5/16-conjugation complex mEGFP-Atg8 was conjugated to the GUV-membrane, while with controls where only mEGFP-Atg8 was added alone to the GUVs, no conjugation has been observed. In the set ups with mEGFP-Atg8 conjugated to the membrane, a tendency of mCherry-Atg34 binding to the GUVs could be observed as well. Still in the entire course of the experiments results were ambivalent and to some extent even the AIM-mutant seemed to be tethered to the membrane. Which would only be expected of wildtype mCherry-Atg34. Binding of mCherry-Atg34 to mEGFP-Atg8 is shown in figure 44.
5.4.2 GUV-assays with DGS-NTA(Ni)-lipids (phase separated GUVs)

Since the use of all the protein components needed for the conjugation process of Atg8 to the membrane, made the experiment very complex and thus variable, a simplified system was created, where N-terminally 6xHis-tagged mEGFP-Atg8 was directly bound to GUV-membranes that contained Ni^{2+}-lipids. Thus the conjugation process was omitted. In these assays mEGFP-tagged Atg8 was bound to the membranes of the GUVs via a 6xHis-tag, that would interact with Ni^{2+}-lipids in the membrane. Here mCherry-Atg34 bound mEGFP-Atg8 on the membrane, while mCherry-Atg34W409A did not. Still to some extent during the course of the experiments binding of Atg34 or Atg34W409A occurred as well when added to GUVs with no Atg8 present. Results are shown in figure 45.
Fig. 45: GUV-assays with membranes containing DGS-NTA(Ni)-lipids. A: Channel-split image of 6xHis-tagged mEGFP-Atg8 bound to Ni\(^{2+}\)-residues of the GUVs followed by mCherry-Atg34 binding to Atg8. B: Binding of mCherry-Atg34 and mCherry-Atg34W409A, respectively, to the GUVs in the absence of Atg8. Scale bars are 10\(\mu\)m.

**5.4.3 GUV-assays with POPC and DGS-NTA(Ni)-lipids**

In these assays again mEGFP-tagged Atg8 was bound to the GUV-membranes via its 6xHis-tag, which interacted with Ni\(^{2+}\)-residues of the membrane-lipids. The GUVs in this set-up, however, where only composed of POPC and DGS-NTA(Ni)-lipids, eliminating the possibility of phase-separation of lipid-constituents. In this reduced set up mCherry-Atg34 was shown to bind to mEGFP-Atg8 on the GUVs (shown in figure 46), while the mutant mCherry-Atg34W409A did not (data not shown).
5.5 GST-Pulldown Assay Using Atg34 and Atg8

To elucidate the interaction of Atg34 and Atg8 in solution GST-pulldown assays were conducted. Samples were taken before incubation and of the unbound prey fractions (unbound Atg8) as well as of bound fractions of the beads and loaded onto 15%-SDS gels. GST (bait) and Atg8 alone were used as controls. As shown in figure 47 no convincing evidence has been found that Atg8 would bind to immobilized full-length Atg34 on the beads. No difference in the binding of Atg8 to Atg34 when compared to the controls could be observed.
Fig. 47: GST-Pulldown Assay. In vitro interaction assay of GST-fused Atg34 and Atg34W409A, respectively with Atg8 (A). Input, unbound prey-fraction and interacting proteins bound to Sepharose-beads were subjected to SDS-PAGE and detected by Coomassie Blue staining. Free GST incubated with Atg8 and Atg8 alone were used as controls (B). No interaction between immobilized GST-Atg34 and Atg8 was detected under these conditions.
5.5 Complexation Experiment by Analytical Size Exclusion Chromatography

In this experiment the concentration dependence of the Atg34-Atg8-interaction was tested, therefore the behavior of both proteins was studied under two different conditions – at low concentrations of 40µM each and at high concentrations of 740µM each. Corresponding SEC- data and SDS-gels can be found in figures 48 to 51 - these findings indicates that Atg34-Atg8-interactions depend on high concentrations and where observed here at 740µM, while hardly any binding of both proteins took place at low concentrations of 40µM.
Fig. 48: Complexation-experiment at low protein concentrations. Diagram-overlay: 40μM of Atg8 and Atg34 have been loaded onto the column (Superose 6 3.2/30).
Fig. 49: SDS-PAGE of gel-filtration samples at low protein concentrations. Peak fractions were collected and loaded onto 15% SDS-gels. After SDS-PAGE protein bands were detected by Coomassie blue-staining. No interaction of Atg34 with Atg8 at this concentration in solution is visible.
Fig. 50: Complexation-experiment at low protein concentrations with controls. Diagram-overlay: 40µM of Atg8 and Atg34W409A have been loaded onto the column (Superose 6 3.2/30).
Fig. 51: SDS-PAGE of gel-filtration samples (control) at low protein concentrations. Peak fractions were collected and loaded onto 15% SDS-gels. After SDS-PAGE protein bands were detected by Coomassie blue-staining. No interaction of Atg34W409A with Atg8 in solution is visible. Fig. 51: Complexation-experiment at high protein concentrations. Diagram-overlay: 740µM of Atg8 and Atg34 have been mixed and loaded onto the SEC-column (Superose 6 3.2/30).
Fig. 52: Complexation-experiment at high protein concentrations. Diagram-overlay: 40µM Atg8 alone, 40µM Atg34 alone and 740µM of Atg8 plus Atg34 have been loaded onto the column (Superose 6 3.2/30).
Fig. 53: SDS-PAGE of gel-filtration samples at high protein concentrations. Peak fractions were collected and loaded onto 15% SDS-gels. After SDS-PAGE protein bands were detected by Coomassie blue-staining. Interaction of Atg34 with Atg8 is visible at this concentration in solution.
Fig. 54: Complexation-experiment at high protein concentrations using Atg34W409A as a control. Diagram-overlay: 40µM of Atg8 alone, 40µM of Atg34 alone and 740µM of Atg8 plus Atg34W409A have been loaded onto the column (Superose 6 3.2/30).
Fig. 55: SDS-PAGE of gel-filtration samples (control) at high protein concentrations. Peak fractions were collected and loaded onto 15% SDS-gels. After SDS-PAGE protein bands were detected by Coomassie blue-staining. No interaction of Atg34W409A with Atg8 in solution is visible.
Fig. 56: Overlay of both Atg8 plus Atg34 and Atg8 plus Atg34W409A-interactions in solution at high protein concentrations (740µM). A clear shift of the curve can be seen were the Atg34-Atg8-mix was loaded onto the column indicating that due to their interaction both proteins eluted as one complex, while no interaction of Atg8 with the mutant was discovered.
6. DISCUSSION

In the course of this work, experimental data was gained, that further indicates an interaction between the two proteins Atg8 and Atg34, as has been stated in former reports [13]. This interaction most likely enables the cargo-uptake into the vacuole in selective autophagy under starvation conditions in yeast [13]. Since this interplay is inhibited, when a mutation in the Atg8 family-interacting motif in the C-terminal region of Atg34 is present, the AIM is believed to be crucial for the binding of the two proteins. Data gained from GST-pulldown assays indicates that other structural components might play a part in this interaction as well, because no interplay of both proteins was detected when Atg34 was immobilized while Atg8 was free in solution (figure 47). Furthermore the findings of this work suggest, that Atg8-Atg34-interactions depend on high concentrations (up to 740µM) of both proteins (figure 52 to 55). Still Atg34 has only been identified in 2010 and since the outcome of this work in parts is contradictory – no interaction between Atg34 and Atg8 could be observed with GST-pulldown assays and definite results were gained with the GUV-assays - more research on this protein is indispensable to further elucidate its characteristics and properties.

6.1 Expression of Atg34-fusion proteins is influenced by their fluorescence-tag

Test expressions of the created 6xHistidine-tagged mCherry-Atg34 and mEGFP-Atg34-gene constructs revealed different ideal expression conditions for each protein. The possibility of inclusion body formation is a known problem of *E. coli*, especially with proteins consisting of >100 residues. One common strategy to avoid protein aggregation is decreasing the growing temperature and thus decelerate transcription and translation rates and thereby diminishing hydrophobic interactions leading to protein misfolding [16, 24]. Consistently, soluble mCherry-Atg34 is best expressed at 25°C over night, while mEGFP-Atg34 expression at 18°C for 5h was ideal to gain soluble protein. Since only the mCherry-protein versions were needed for later experiments, large scale expression was only continued with these constructs.

6.2 Atg8 does not bind immobilized full-length Atg34 in solution

In the GST-pulldown assays, when N-terminally tagged GST-Atg34 was bound to
Glutathione Sepharose 4B-beads (GE Healthcare) and probed with Atg8, no compelling interaction of both proteins has been observed (figure 47). These findings might suggest, that other structural components of Atg34 would further facilitate the protein-protein-interactions under cellular conditions, which might have been hidden or hindered by the immobilization on the beads. Furthermore under natural conditions, Atg8 is first conjugated to the PAS [8] and therefor is locally concentrated there while Atg34 is free in solution – thus in this assay the natural conditions were inverted. This could be another reason for the protein-binding to fail. Besides, the data gained in the analytical SEC-experiments suggest that Atg8 and Atg34 would only interact when high protein amounts (up to 740µM) of each protein are available. Thus the concentrations used in the GST-pulldown assays (12.5µM) could simply have been too low to enable any interaction.

6.3 Atg34 is recruited to the membrane via Atg8-interactions

In-vivo Atg8 is activated by Atg3 and Atg7 and then conjugated to phosphatidylethanolamine in the isolation membrane where the conjugation-complex, constituted of Atg12-5-16 with Atg3 and Atg7 functioning as E1 and E2-like enzymes, facilitates the reaction by tethering Atg8 to the membrane. There, at the membrane Atg34 would bind to Atg8 to tether its cargo to the isolation membrane. In the course of this work, these mechanisms have first been investigated by GUV-assays using all of these protein components. Data gained indicated that the Atg8-Atg34 interactions take place on the membrane (figure 44) and that these interactions would depend on the AIM-motif at the C-terminus of Atg34 – as was hypothesized before. Still, to a lower extend, binding was observed as well using the Atg34W409A-mutant. However, using all of these components made the very complex and thus variable. Thus a simplified system was created, where 6xHis-tagged mEGFP-Atg8 was directly bound to GUV-membranes that contained Ni$^{2+}$-lipids and the conjugation process was therefore omitted. Starting with phase separated GUVs (which were used by other members of the group and therefore available) Atg8-Atg34 interactions were again observed, while no binding occurred when the AIM-mutant of Atg34 was used. Occasionally in experimental set ups where no Atg8 was present at all, binding of both Atg34 or Atg34W409A would be seen (figure 45). This most likely happened due to the phase separation of membrane-lipids leading to spots of imbalanced high concentrations of Ni$^{2+}$-
lipids in the GUVs, that would interact with residual 6xHis-tags on Atg34 or Atg34W409A, which probably outlasted the purification process. However, in the presence of Atg8, it was preferably bound to the GUVs, indicating a higher binding affinity of this protein. Irregularities in the observed protein-binding, could also have been caused by varying light exposure times during microscopy. To omit phase separation of membrane lipids in the GUVs, another lipid-mixture was created, that solely consisted of POPC-lipids and DGS-NTA(Ni)-lipids providing the Ni$^{2+}$-lipids for the binding of 6xHis-Atg8. With these GUV-assays the binding of Atg34 to Atg8 has been shown (figure 46), while no binding occurred when the AIM-mutant Atg34W409A was used. Here, in the absence of Atg8, no binding of Atg34 or Atg34W409A, respectively, has been observed. These findings suggest that Atg8 has to be present on the isolation membrane to allow Atg34 to bind to the membrane and that this protein-protein-interaction depends on a functional AIM-motif at the C-terminus of Atg34.

6.4 Atg8-Atg34-Interactions Depend On High Concentrations of Both Proteins
Data gained from the complex-formation experiment by analytical SEC provides evidence that the interaction of Atg8 with Atg34 in solution requires high concentrations of both proteins (up to 740µM). This might be a form of regulation mechanism for yeast cells to induce the Atg34-dependent autophagy pathway only when Atg34 is up-regulated upon nutrient starvation. Or that interactions only take place at the isolation membrane where Atg8 is locally concentrated.
7. Summary

Autophagy represents a lysosomal pathway, which is induced upon stress and empowers cells to turn over cellular organelles and proteins to reuse them as a source of energy. It plays a key role in a variety of diseases and can either be non-selective or selective. In the process of (selective) macroautophagy a phagophore is created initially in which cytosolic cargo becomes incorporated. The phagophore then matures and fuses with the lysosome (mammals), or the vacuole (yeast and fungi) to form the autolysosome, in which proteolytic degradation takes place.

In yeast autophagy is regulated by Autophagy related genes (Atg). One of them is Atg34 – a cargo-receptor protein that appears in a sidearm of the Cvt-pathway. To incorporate Ams1 into the autophagosome Atg34 comprises a C-terminal Atg8 family-interacting motif (AIM) to interact with Atg8 on the autophagosomal membrane. It was the aim of this work to further elucidate the mode of interaction of Atg34 with Atg8 either in solution or at the autophagosomal membrane. The interaction of both proteins at the membrane was studied using Giant Unilamellar Vesicles (GUVs) in different fluorescence-microscopy assays, providing evidence that Atg8 has to be present at the membrane to recruit Atg34 and that this interaction depends on a functional AIM.

The interaction of both proteins in solution was tested with GST-pulldown assays and analytical gel filtration. In the GST-pulldown assays Atg34 has been coupled to Glutathione Sepharose-beads upon a GST-tag and incubated with free Atg8. Here no interaction between Atg34 and Atg8 has been observed. With the gel filtration experiment the concentration dependence of the Atg34 and Atg8 interaction has been studied. Results provide evidence that the interplay in solution needs high concentrations (up to 740µM) of both protein partners. Still, additional research to clarify cargo-recruitment to the autophagosomal membrane will be necessary.

7.1 Zusammenfassung

Autophagie ist ein lysosomaler Stoffwechselweg, der durch Stress induziert wird und es Zellen ermöglicht, Organelles und Proteine umzubauen, um sie als Nährstoffquelle zu nutzen. Sie spielt eine Schlüsselrolle bei vielen Erkrankungen und kann unselektiv oder selektiv sein. Bei der (selektiven) Makroautophagie wird zuerst eine Phagophore gebildet, in die cytosolischer Cargo eingebaut wird. Die
Phagophore reift und fusioniert mit dem Lysosom/ der Vakuole, um das Autolysosom zu bilden, wo der proteolytische Abbau stattfindet.


8. Literature

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38. Internet, 2013: http://www.snapgene.com/
# 9. Curriculum Vitae

## Personal Details

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## Education

| Since 04/10 | Master studies in Biochemistry and Molecular Biomedicine at Graz University  
Specialisation on molecular biomedicine and cell biology |
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<td>Additional studies in Biology at the University of Vienna</td>
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## Research Experience

| 10/11 – 04/12 | Master's Thesis at the University of Vienna (Vienna Biocenter,  
Max F. Perutz Laboratories) at the Department of Biochemistry and Cell Biology  
Titel of thesis: Atg34 in Selective Autophagy  
Supervisors: Professor Dr. Graham Warren and Dr. Sascha Martens |
|---------------|-----------------------------------------------------------------------------|
| 08/11 – 10/11 | Project work at the Department of Molecular Microbiology at Graz University  
Description: studying the accumulation of beta-amyloid-peptides in Alzheimer's disease |