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UNIVERSITY OF CALIFORNIA, SAN DIEGO

A Common Model for Phospho-regulation of Pexophagy and Mitophagy Receptors

A Thesis submitted in partial satisfaction of the
Requirements for the degree of Master of Science

in

Biology

by

Aaron Burkenroad

Committee in charge:

Professor Suresh Subramani, Chair
Professor Goran Bozinovic
Professor James Wilhelm

2014

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Chair

University of California, San Diego

2014

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ACKNOWLEDGEMENTS

Without the support and guidance of many people, this work would not have been possible. First and foremost, I am eternally grateful for the unending assistance and constant support of my mentor, Dr. Jean-Claude Farré. Thank you for your patience in answering my questions at every step of the way and teaching me every experimental and analytical technique I now know today. With your mentorship I have learned what it means to truly think critically, consider alternative explanations, and become actively engaged in the research process. It has been a privilege to collaborate with you during my time in the lab. I would like to especially thank Dr. Suresh Subramani for inviting me to work in this lab and giving me the opportunity to participate in the Contiguous BS/MS Program. I would also like to extend my gratitude to the (soon to be Dr.) Sarah Burnett. As my permanent bench mate she not only provided me with her company whether I wanted it or not, but offered her helpful opinions and assisted with me with several experiments that you will see presented in this work. The previous work and expertise of previous and current members of the Subramani lab, as well as some of their strains, plasmids, and reagents, have been very helpful as I carried out this project. I am grateful for the support and good company of all present and past members of the lab who I had the pleasure to meet over these two years. Finally, I would like to thank my family and friends for their unwavering support and understanding of my efforts in the lab and other pursuits I have chosen to take.

Portions of this thesis contain data and findings that have been published in a 2013 EMBO Reports article titled “Phosphorylation of pexophagy and mitophagy receptors coordinates their interaction with Atg8 and Atg11” [*EMBO Rep.* 14(5): 441-9]. The corresponding author Dr. Suresh Subramani and co-authors Dr. Jean-Claude Farré and Sarah Burnett have approved the inclusion of these findings in this work.

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ABSTRACT OF THE THESIS

A Common Model for Phospho-regulation of Pexophagy and Mitophagy receptors

by

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Master of Science in Biology

University of California, San Diego, 2013

Professor Suresh Subramani, Chair

Organisms must find a way to degrade and recycle cytoplasmic contents and eliminate redundant organelles. Peroxisomes and mitochondria undergo rapid, targeted degradation in eukaryotic cells by the tightly-regulated pathways of pexophagy and mitophagy, respectively, when they become redundant, damaged, or when the cell experiences starvation or stressful conditions. As classical selective autophagy receptors,

Atg19 (yeast receptor for the cytosol to vacuole targeting pathway) and Atg32 (yeast mitophagy receptor) interact with the autophagy-related proteins, Atg8 and Atg11. We identified a novel Atg8-family interacting motif (AIM) of the *Saccharomyces cerevisiae* pexophagy receptor, Atg36, that is required for its interaction with Atg8. We also found that both Atg32 and Atg36 contain phospho-regulated sites that are required for their interactions with Atg8 and Atg11. Each Atg36 molecule must interact with both Atg8 and Atg11 in order to be fully functional in mediating pexophagy. In this work, we present a common model for the phosphoregulation of selective autophagy receptors. Furthermore, we demonstrated that the kinase, Slt2, previously known to regulate selective autophagy pathways, plays a key role in the phosphorylation of both Atg32 and Atg36. This discovery has led to a deeper understanding of the signaling, regulation, and molecular mechanisms of selective autophagy pathways that may apply to how these processes occur in higher eukaryotes.

I. Introduction

Autophagy

In order to function in and respond to a diverse range of environmental conditions, living organisms must rely on an essential set of mechanisms that control internal parameters such as temperature, pH, and metabolic rate. Organisms must also find a way to degrade and recycle superfluous or damaged organelles and other cytoplasmic constituents in order to promote and maintain cellular homeostasis. Autophagy, an intracellular pathway by which a cell degrades its own contents, occurs in virtually all eukaryotes,. Through the use of electron microscopy, Christian de Duve, who coined the term “autophagy” (literally “self eating”), showed single- or double-membrane vesicles containing portions of cytoplasm and organelles in mammalian cells (de Duve and Wattiaux, 1966). During autophagy, cells can sequester cytoplasmic materials and interior cell structures to be broken down and recycled via lysosomal or vacuolar degradation through two distinct mechanisms, macroautophagy and microautophagy.

In macroautophagy, these large cargos are engulfed by double-membrane structures known as autophagosomes, targeted to the lysosome (or vacuole in yeast), and recycled back into the cytosol (Klionsky and Ohsumi, 1999). On the other hand, microautophagy is a process in which the lysosomal or vacuolar membrane invaginates and directly engulfs cytosolic components that are then degraded (Figure 1-1). Autophagy is a tightly regulated and non-selective process employed to generate macromolecular building blocks, such as amino acids, and energy required to meet minimum survival requirements during periods of nutrient deprivation and stress. Autophagy is also important for the removal of damaged and superfluous organelles, protein aggregates or misfolded proteins, and other cytoplasmic contents.

Implications of autophagy in human physiology and disease

Autophagy is now known to play a role in a variety of human disease conditions. A correlation between autophagy and human disease was first shown by Liang *et al.*, who demonstrated that single allele deletions of the *BECN1/ATG6* gene caused mice to be at risk for spontaneous tumor formation (Liang *et al.*, 1999). Since this discovery, numerous correlations have been shown between dysfunctional autophagic processes and disease conditions such as neurodegenerative disorders and cancer.

For example, double deletion of the *ATG5* gene specifically in neuronal cells, which is required for autophagosome formation, results in the aggregation of cytoplasmic proteins and deficits in neuronal function (Hara *et al.*, 2006). Inhibition of autophagy in cells expressing a mutant protein associated with Huntington's disease caused increased aggregation and cell death (Ravikumar *et al.*, 2002). These and other findings suggest that a basal level of autophagy is required for clearance of protein aggregates in neurons to prevent neurodegenerative disorders such as Alzheimer's and Huntington's.

Furthermore, studies have suggested a role of autophagy as a tumor suppressor by protecting cells from DNA damage that would otherwise result in oncogenesis (Mathew *et al.*, 2007). Autophagy has also been shown to be a necessary adaptation for post-mitotic cells, such as cardiac myocytes, to survive and tolerate stresses (Nakai *et al.*, 2007). Enhanced activity of mTORC1 (mTOR Complex 1) in podocytes, which is associated with inhibition of autophagy, causes many phenotypes seen in human Diabetic Nephropathy (DN). A reduction of mTORC1 expression in podocytes prevents DN in diabetic animal models (Gödel *et al.*, 2011; Inoki *et al.*, 2011). Clearly, autophagy has been implicated in a variety of disease conditions and is important for cell growth and

survival, immune and nervous system integrity, tumor suppression, and apoptosis. Any defects or dysregulation of the autophagic pathway could have significant consequences for human development, survival, and incidence of a wide range of diseases.

Autophagy – general mechanism and regulation

Molecular investigation into the autophagy process began with genetic screens of budding yeast such as *S. cerevisiae*, *Pichia pastoris*, and *Hansenula polymorpha*, which have led to identification of autophagy-related proteins that are encoded by the *ATG* genes (He and Klionsky, 2009). Importantly, the majority of these genes was shown to be conserved from yeast to humans (Klionsky *et al.*, 2003), which demonstrates the significance of the autophagy mechanism for survival and viability. Studies using *S. cerevisiae* as a model have determined the steps involved in the mechanism of autophagy and autophagy-related pathways. The steps involved in macroautophagy in yeast are the following:

- 1) Signaling and induction of autophagy: carbon, nitrogen, phosphate, or sulfate starvation causes induction of autophagy. Tor1, a protein kinase that regulates cell growth in response to nutrient availability and cellular stress, hyperphosphorylates Atg13 when nutrients are abundant. This hyperphosphorylation event prevents autophagy by blocking the interaction of Atg13 with Atg1 (Nair and Klionsky 2005). Upon starvation, the Tor1 pathway is inactivated, resulting in rapid partial dephosphorylation of Atg13 and activation of Atg1 kinase activity (Kamada *et al.*, 2000).

- 2) PAS formation: A complex of proteins aggregates at the phagophore assembly site (PAS), where eventual membrane elongation and recruitment of cargo occur. A variety of Atg proteins assemble at the PAS: the Atg1 kinase complex, the Atg2-Atg18 complex, the Atg8 and Atg12 systems, the phosphatidylinositol 3-kinase (PI3K) complex, and Atg9, a transmembrane protein that shuttles between the PAS and peripheral sites (Suzuki and Ohsumi, 2007; Klionsky 2005).
- 3) Vesicle formation: Atg8 and Atg12 are ubiquitin-like proteins which become conjugated to phosphatidylethanolamine (PE), a phospholipid that makes up the vesicular membrane, and Atg5, respectively (Abeliovich *et al.*, 2000). This step in the process involves many Atg proteins and is necessary for expansion of the membrane around the selected cargo.
- 4) Retrieval: Several components of the autophagy machinery are then recovered for recycling and reuse in subsequent cycles of vesicle formation. Atg8 associates with PE on the membrane of the expanding phagophore, but is cleaved from PE on the outer phagophore membrane as the tips of the enlarging phagophore fuse to form a double-membrane vesicle known as the autophagosome. Inability to cleave off Atg8 from PE at this late stage of autophagosome formation causes a pexophagy defect (Kirisako *et al.*, 2000). Most Atg proteins also dissociate from the

autophagosomes. Likewise, Atg9, the only integral membrane-associated protein, cycles between the PAS and a peripheral compartment, the potential membrane source for autophagy. Thus much of the core autophagy machinery is recruited to the PAS to facilitate phagophore elongation, but upon completion of the autophagosome, these components are recycled back for further use.

- 5) Vesicular docking and fusion with the vacuole: Once vesicle formation is complete, the vesicle can fuse with the vacuole and efficiently release cargo. Many proteins that also function in other targeting pathways also participate in vesicle fusion during autophagy and autophagy-like pathways, such as the SNARE proteins and class C Vps/HOPS complex proteins.
- 6) Breakdown of the intraluminal vesicle and its cargo and recycling of the macromolecular constituents: Following fusion of the vacuole with the outer autophagosomal membrane, the single-membrane vesicles containing cargo termed autophagic bodies must be degraded. The acidity within the vacuole and Atg15, a lipase, are thought to induce breakdown of the single membrane, releasing the cargo into the vacuolar lumen. Vacuolar proteases, proteinase A (Pep4) and proteinase B (Prb1), degrade the cargo into its constituents (Klionsky 2005; Farré and Subramani 2004; Takeshige *et al.*, 1992). Finally, Atg22 and other amino acid

transporters export amino acids back into the cytosol, where they can be reused (Yang *et al.*, 2006).

Selective Autophagy

Autophagy has been described as a degradative pathway that results in the non-selective, bulk degradation of cytoplasmic contents. In yeasts, this nonselective form of autophagy mainly serves as a survival response in starvation conditions, providing a source of amino acids and other nutrients to maintain cell viability. However, researchers first demonstrated in 1973 that sequestration of smooth endoplasmic reticulum could be achieved via an autophagy-like pathway (Bolender and Weibel, 1973). Sequestration of mitochondria and peroxisomes was also observed (Beaulaton and Lockshin, 1977; Veenhuis *et al.*, 1983). The cytoplasm-to-vacuole targeting pathway (Cvt) is an autophagy-like biosynthetic pathway in which vesicles transport specific cargos, such as the precursors of the vacuolar hydrolases aminopeptidase 1 (Ape1) and α -mannosidase (Ams1), to the vacuole. The Cvt pathway relies on the function of many of the same proteins as the autophagy pathway, but also requires specific proteins to mediate its specificity. The Ape1 precursor forms dodecamers, which aggregate into a larger Ape1 complex. Atg19, which serves as the Cvt-specific receptor protein, binds this Ape1 complex and recruits it to the PAS through the interaction with scaffold protein, Atg11 (Kim *et al.*, 2001). Atg19 also binds to Atg8, which causes membrane elongation and engulfment of the cargo into a vesicle. Similarly, other forms of selective autophagy each require their own specific selectivity protein/s in order to interact with the core autophagic machinery proteins.

Selective autophagy can effectively mediate cellular homeostasis by tightly regulating the turnover of target organelles and other cytoplasmic structures, such as peroxisomes (pexophagy), mitochondria (mitophagy), ribosomes (ribophagy) and ER (ER-phagy). Each of these selective autophagy mechanisms, like the Cvt pathway, employ many of the genes involved in autophagy and also require their own specific adaptor proteins (Johansen and Lamark, 2011).

Yeast as a model organism for the study of selective autophagy pathways

The use of yeast as a model organism for the study of myriad biological processes has a long history. The first complete DNA sequence of a eukaryotic genome was completed for the budding yeast *S. cerevisiae* in 1996. This effort has proven to be an indispensable tool for the study of human biology, as the yeast genome encodes many proteins that resemble those found in mammals (Botstein and Fink, 1988). Surprising links between the specific sequence and function of human and yeast genes, making the yeast model system very useful model for the study of human disease.

One of the most intriguing examples of this homology was the finding that yeast lacking both RAS1 and RAS2 genes in yeast, close homologues of the *ras* proto-oncogene in mammals, are nonviable mutants. When the mammalian H-*ras* gene was expressed in yeast lacking both genes, viability was restored, indicating a surprising conservation of sequence and function (Kataoka *et al.*, 1984). In addition, yeasts can be easily and cheaply manipulated by various genetic techniques, and their short generation time and conservation of mechanisms contribute to their utility as a model for the study

of biological processes. In addition, the yeast two-hybrid method has been used extensively in this work to reveal details of various protein interactions.

Yeasts are especially useful for the study of autophagy and autophagy-related pathways. From the initial discovery in the early 1990s that autophagy occurs in yeast, our understanding of autophagy has improved dramatically. Methylophilic yeasts such as *P. pastoris* and *H. polymorpha* are particularly useful for the study of peroxisome homeostasis, because manipulation of nutrient composition can stimulate both peroxisome proliferation and degradation. Media containing methanol can promote peroxisome proliferation. When these yeasts are shifted to media containing glucose or ethanol, peroxisomes are no longer required for energy production, causes induction of peroxisomal degradation by macro- or micropexophagy.

The use of *S. cerevisiae* has also been useful in the study of selective autophagy; genetic screens of this species have resulted in the discovery of many of the *ATG* genes required for general and selective autophagy. Furthermore, *S. cerevisiae* employs the same signaling cascades that regulate cellular metabolism in response to environmental stressors and nutrient availability in higher eukaryotes, and these pathways are thought to be imperative for regulation of autophagic processes. The study of selective autophagy signaling and regulation is the major focus of this work.

Peroxisome Homeostasis: A balance between biosynthesis and selective degradation

Peroxisomes are single membrane-bound organelles found in virtually all eukaryotic cells and participate in a variety of metabolic and anabolic pathways. They carry out both α - and β - oxidation of fatty acids and synthesis of bile acids and

cholesterol (Van Veldhoven, 2010). Additionally, peroxisomes are essential in mammals for the catabolism of long-chain fatty acids, D-amino acids, and plasmalogens which are essential for proper brain and lung function (Wanders and Waterham, 2006). They are also essential for cellular protection, by scavenging toxic and reactive by-products of oxidative reactions. These single membrane-bound organelles range in size from 0.1 to 1.0 μm , and their subcellular distribution and number vary widely between organisms and depend heavily on environmental conditions and nuclear availability. In human beings, defects in at least 12 of the *PEX* genes involved in peroxisome biosynthesis and other resident peroxisomal proteins can lead to devastating disorders in the Zellweger syndrome spectrum (PBD-ZSS) (Steinberg *et al.*, 2004). The balance between peroxisome biogenesis and turnover by selective autophagy is essential for maintenance of metabolic homeostasis.

Peroxisomes are distinct from mitochondria and chloroplasts in that they do not contain their own DNA. Two models currently exist to explain how peroxisome biogenesis occurs: the “growth and division” model (Purdue and Lazarow, 2001a) or *de novo* biogenesis from the endoplasmic reticulum (ER) (Hoepfner *et al.*, 2005). Electron microscopy studies revealed that peroxisomes were closely associated with the ER, suggesting that peroxisomes might originate from the ER (Novikoff and Novikoff, 1972; Geuze *et al.*, 2003). However this model was poorly accepted due to a lack of biochemical evidence. Subsequent studies of peroxisome biogenesis found that peroxisomal enzymes and peroxisomal membrane proteins (PMPs) were synthesized on free polyribosomes and imported post-translationally into the organelle (Rachubinski *et*

al., 1984). These findings provided evidence for the “growth and division” model of peroxisome biogenesis.

However, subsequent mutagenesis studies in which several *PEX* gene mutants ($\Delta pex3$ or $\Delta pex19$) effectively blocked peroxisome assembly in yeast and mammalian cells. Upon reintroduction of the missing wild-type genes in these mutant fibroblast and yeast cells lacking peroxisomes, this organelle could be reformed *de novo* (Hohfeld *et al.*, 1991; Subramani, 1998). Further biochemical evidence of the *de novo* biogenesis model came from a recent study in which 16 PMPs encompassing all types of membrane topologies were shown to first target to the ER and translocate to peroxisomes (van der Zand 2010). According to this model PMPs are co-translationally targeted to the ER and sorted to a specialized domain known as the pre-peroxisomal compartment. This compartment is then budded from the ER to form pre-peroxisomal vesicles in a Pex19p-dependent manner (Agrawal *et al.*, 2011; Lam *et al.*, 2010).

Peroxisomal matrix are targeted to peroxisomes via one of two targeting signals: PTS1 or PTS2. PTS1- and PTS2-containing proteins are recognized and bound by the receptor proteins Pex5 and Pex7, respectively (Hettema *et al.*, 2000; Subramani, 1998). These receptor-cargo complexes are then recruited to the peroxisomal membrane, where they interact with the docking subcomplex of the importomer (Hazra *et al.*, 2002). This interaction induces translocation of the entire complex across the membrane, where cargos are released and PTS receptor complex proteins are recycled to repeat the process of cargo recruitment and translocation (Ma and Subramani, 2009).

Pexophagy has been found to occur in many organisms from single-celled eukaryotes to mammals but has been most extensively studied in yeasts. It was first

noted when peroxisomes were observed in the lysosomes of mammalian liver cells (de Duve and Baudhuin, 1966). As mentioned previously, each selective autophagy pathway requires a specific cargo receptor that mediates interaction between the selected cargo and the universal autophagy machinery. In *P. pastoris*, a methylotrophic yeast, Atg30 serves as the pexophagy receptor. Atg30 interacts with Pex3 and Pex14 on the peroxisomal membrane, with another pexophagy-specific protein Atg37, as well as with Atg11 and Atg17 of the core autophagic machinery. $\Delta atg30$ and $\Delta atg37$ cells exhibit a full block in pexophagy, and overexpression of Atg30, but not Atg37, in conditions that promote peroxisome proliferation, induces pexophagy (Farré *et al.*, 2008). The cargo receptor protein usually has a tripartite role in selective autophagy: (1) cargo binding, (2) interaction with Atg11, a protein that serves as a scaffold to facilitate the formation of the PAS and (3) interaction with Atg8 via an AIM. However, direct interaction between Atg30 and Atg8 had not been observed. Atg36 was recently shown to be the pexophagy receptor in *S. cerevisiae* and perform all three of these functions, similarly to Atg19 of the Cvt pathway and Atg32 of mitophagy (Motley *et al.*, 2012). Further study into the molecular mechanisms of pexophagy and its receptors in yeast will lead to a better understanding of these pathways are regulated.

Introduction to mitophagy

Mitochondria are essential for providing eukaryotic cells with a continuous supply of energy in the form of ATP. However, these organelles can quickly produce a toxic intracellular environment via excessive release of reactive oxygen species (ROS), and can subsequently release proteins that promote cell death. In order for the eukaryotic cell to

protect the cell against harm or cell death pathways such as apoptosis or necrosis, it can activate the selective degradation of aberrant mitochondria, or mitophagy. The balance between cell death and mitophagy is a tightly regulated process that has opposite effects on cell survival. Like other forms of selective autophagy, mitophagy is an evolutionarily conserved mechanism that plays an essential role in maintaining cellular homeostasis and integrity.

Mitophagy has also recently been implicated in a variety of human diseases. Several studies suggest that mitophagy promotes tumorigenesis by maintaining a healthy pool of mitochondria that is capable of meeting the energy demands of tumor cells (Gou *et al.*, 2011). A link has also been found between Parkin, a protein implicated in Parkinson's disease and encoded by the PARK2 gene in humans, and aspects of the mitophagy pathway (Geisler *et al.*, 2010). Deregulation of mitochondrial dynamics is clearly associated with various human disease states such as cancer and Parkinson's disease. Further investigation into mechanisms and regulation of mitophagy can help to shed light on these disease processes and support the development of new therapies.

Atg32, which localizes on mitochondria in *S. cerevisiae*, is the specific receptor that facilitates selective degradation of mitochondria by binding to Atg11 and Atg8 following induction of mitophagy (Kanki *et al.*, 2009; Okamoto *et al.*, 2009). Further studies have revealed that Atg32 shares many of the same functional characteristics of Atg19, the "classic" receptor protein of the Cvt pathways (Okamoto *et al.*, 2009). However, further study into the molecular mechanisms and regulation of mitophagy can lead to discoveries that can be applied to the process in yeast and higher eukaryotes.

Mechanisms of regulation of pexophagy and mitophagy

The classic selective autophagy receptors, Atg19 and Atg32, confer selectivity by binding their respective cargos and interacting with proteins of the core autophagic machinery, Atg8 and Atg11, at specific sites. These receptors interact with Atg8 via AIMs (Atg8- family Interacting Motifs) in yeast and LC3-interacting regions (LIRs) in mammals, which have a WxxL-like consensus sequence required for Atg8 or LC3 binding (Noda *et al.*, 2010). Atg19 possesses such a motif near its C-terminal end (Shintani *et al.*, 2002), and Atg32 possesses an AIM near its N-terminus (Okamoto *et al.*, 2009b). Selective autophagy receptors also interact with Atg11 via a conserved binding site that contains a Serine residue. The sequences of several selective autophagy receptor homologues reveal a highly conserved sequence for Atg11 binding that closely follows the (D/S)ILSSS sequence (Farré *et al.*, 2013).

Atg30 and Atg36 serve as the pexophagy receptors in *P. pastoris* and *S. cerevisiae*, respectively, while Atg32 serves as the mitophagy receptor in both yeast species. These receptors, like Atg19 of the Cvt, interact with Atg11 of the autophagic machinery. In order for this interaction to take place, phosphorylation of each protein is required at specific sites. Phosphorylation of Atg30 at Serine 112 is required for its interaction with Atg11 (Farré *et al.*, 2008). Similarly, phosphorylation of Serine 114 on Atg32 is required for its interaction with Atg11 (Aoki *et al.*, 2011).

Although much has been discovered about how these selective autophagy receptors interact with Atg8 and Atg11, many details of this mechanism and how it is regulated remain unknown. Induction of pexophagy and mitophagy requires the action of protein kinase(s) on specific sites of the receptors Atg30 and Atg32 to facilitate the

interaction with the scaffold protein Atg11. The existence of a similar mechanism to mediate the interaction of Atg30, Atg32, and Atg36 with Atg8 has not been described. Also, questions remain as to how interactions between the receptor proteins and Atg8 and Atg11 proceed: whether they occur sequentially or simultaneously, whether they occur on the same molecule, and whether these modes of interaction are shared by receptors in other selective autophagy pathways. Importantly, the specific kinase(s) that phosphorylate these receptors at their Atg8 and Atg11 interaction sites remain to be discovered.

Implication of kinases in autophagy regulation

In total, 106 protein kinases have been identified in the entire fission yeast genome (Bimbo *et al.*, 2005). The task of identifying which of these kinase(s) is/are involved in regulation of selective autophagy pathways such as pexophagy and mitophagy is complex. As of yet, few kinases have been implicated in the direct phosphorylation of selective autophagy receptors. It was found that TANK Binding Kinase 1 (TBK1) phosphorylated Serine-177 of optineurin, a selective autophagy receptor involved in the autophagic clearance of cytosolic *Salmonella enterica* pathogen. This phosphorylation event enhances optineurin's affinity for LC3 (the mammalian Atg8 homolog) (Wild *et al.*, 2011). Identification of the kinase that performs such a function provides invaluable insight into how the process of selective autophagy of *S. enterica* is triggered and regulated.

While the signaling events that regulate pexophagy and mitophagy are still poorly understood, growing evidence points to intracellular signaling pathways. Recently, there

have been several studies that suggest a role for Mitogen-activate protein kinases (or MAPKs) in autophagy. MAPKs are evolutionarily conserved serine/threonine-specific kinases that act in response to extracellular growth factors, or mitogens. MAPKs are important for a wide variety of cellular processes, such as the cell cycle, morphogenesis, and survival in times of environmental stress. The mitogen-activated protein kinase p38 was shown to regulate starvation-induced autophagy through its interacting partner p38IP and the transmembrane autophagy protein Atg9 (Kaneda *et al.*, 2009). It was also shown that the MAPK Slt2 is required for pexophagy and mitophagy, but not for other autophagy-related pathways such as Cvt (Manjithaya *et al.*, 2010; Mao *et al.*, 2011). Slt2 was shown to be involved in recruitment of the mitochondria to the PAS during mitophagy (Mao *et al.*, 2011). Interestingly, ERK2, another protein of the MAPK class has also been shown to activate mitophagy in mammalian cells (Dagda *et al.*, 2008). Further investigation into how these and other MAPKs function in regulation of selective autophagy is warranted.

It was recently discovered that Casein Kinase 2 is essential for mitophagy, but not other autophagy-related pathways such as macroautophagy, Cvt, or pexophagy in *S. cerevisiae*. CK2 was shown to be the direct kinase that performs phosphorylation of Atg32 at Serine-114 and Serine-119, facilitating the interaction of Atg32 and Atg11 (Kanki *et al.*, 2013). CK2 is a highly conserved serine/threonine protein kinase involved in a variety of processes such as cell survival, the replication cycle, and the stress response (Montenarh, 2010). This study serves as a model for the implication of a kinase in the phosphorylation of an autophagy receptor in yeast, and highlights the possible importance of casein kinase proteins in other selective autophagy pathways. Casein

Kinases are known to phosphorylate proteins at the consensus sequence S/Tp-X-X-(S/T)p. Interestingly, the pexophagy receptors, Atg30 and Atg36, contain several sequences that mimic AIMs and Atg11 consensus binding sites. ScHrr25, a Casein Kinase I homolog, was shown to possibly interact with Atg11 in high throughput screening. Identification of the kinase(s) that phosphorylate Atg30 and Atg36 will lead to a better understanding of how selective autophagy is regulated in response to changes in environmental and intracellular conditions.

The goal of this study was to address some of the unanswered questions regarding the regulation and mechanisms of selective autophagy receptors by using various molecular, biochemical, and analytical methods. As processes that are conserved in humans, insights into the mechanisms and regulation of pexophagy and mitophagy will help support further study of other selective autophagy pathways and may one day be applied to the development of therapies that treat a wide variety of human diseases.

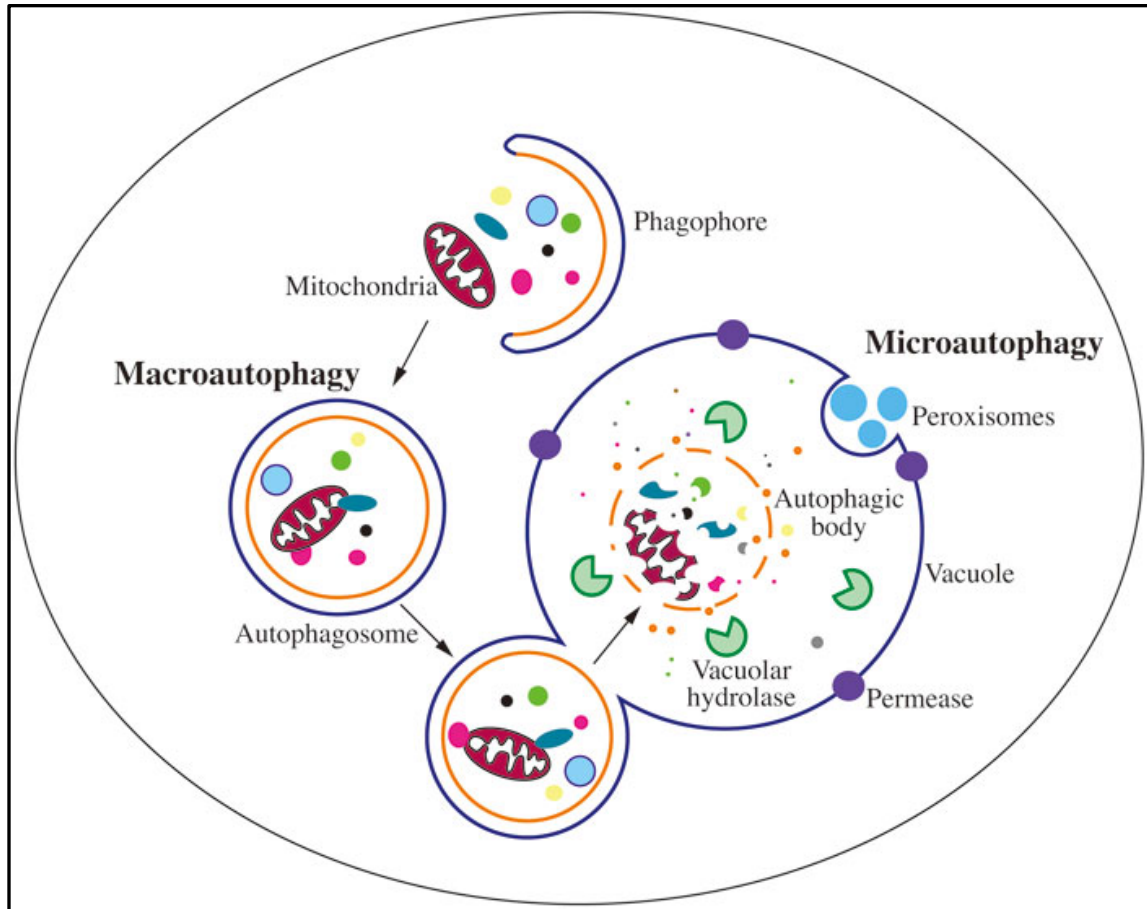


Figure 1-1: Schematic depiction of macro- and microautophagy in yeast. (Feng *et al.*, 2014). In macroautophagy, portions of cytoplasm and dysfunctional or redundant organelles are sequestered by the phagophore membrane, which engulfs these cargos to form the autophagosome. The autophagosome then fuses with the vacuolar membrane, releasing the cargos into the vacuolar lumen to be degraded by vacuolar hydrolases and recycled. In microautophagy, cargos are taken into the lumen of the vacuole by invagination and direct engulfment by the vacuolar membrane, followed by degradation and recycling as it occurs in macroautophagy.

Table 1-1: List of autophagy-related genes [50].

Gene	Description of molecular events	Macropexophagy		Micropexophagy
		<i>Pp</i>	<i>Hp</i>	<i>Pp</i>
<i>ATG1</i>	Serine/threonine kinase required for PAS formation	✓	✓	✓
<i>ATG2</i>	Peripheral membrane protein required for Atg9 recycling	✓		✓
<i>ATG3</i>	E2-like ubiquitin ligase that catalyzes lipidation of Atg8			✓
<i>ATG4</i>	Protease that processes Atg8 as prerequisite for conjugation with phosphatidylethanolamine (PE)			✓
<i>ATG6</i>	Subunit of PI3K complexes I and II	✓		
<i>ATG7</i>	E1-(ubiquitin activating enzyme)-like protein involved in conjugation of Atg12-Atg5 and Atg8-PE conjugates	✓		✓
<i>ATG8</i>	Ubiquitin-like protein that is anchored to the expanded phagophore membrane in its processed and lipidated form, involved in phagophore membrane expansion	✓	✓	✓
<i>ATG9</i>	Transmembrane protein cycling between the PAS and a peripheral compartment	✓		✓
<i>ATG11</i>	Coiled-coil adaptor protein that interacts with the core machinery and known receptors for selective autophagy	✓	✓	✓
<i>ATG16</i>	Essential component of the Atg12-Atg5-Atg16 complex	(✓)		✓
<i>ATG17</i>	Scaffold protein that is responsible for PAS organization	✓		✓
<i>ATG18</i>	PtdIns3P-binding protein whose localization is dependent Atg9 and PtdIns-3P; recruits Atg2 and needed for Atg9 recycling	✓		✓
<i>ATG21</i>	WD40 protein with phosphoinositide binding domain that is involved in pexophagosome formation		✓	
<i>ATG24</i>	Sorting nexin protein involved in fusion events with the vacuole	✓		✓
<i>ATG25</i>	Coiled-coil protein that co-localizes with Atg11 at the PAS, required for macropexophagy		✓	
<i>ATG26</i>	Sterol glucosyltransferase that plays a role in phagophore membrane expansion	✓		✓
<i>ATG28</i>	Coiled-coil protein required for peroxisome sequestration during micropexophagy and vacuole fusion of pexophagosomes in macropexophagy	✓		✓
<i>ATG30</i>	Pexophagy receptor that interacts with peroxins, Pex3 and Pex14, and adaptor proteins, Atg11 and Atg17	✓		✓
<i>ATG35</i>	Localizes to the perinuclear structure; regulates MIPA formation and interacts with Atg28 and Atg17			✓
<i>GCN1-4</i>	Involved in general amino acid control			✓
<i>Sar1</i>	Sec protein required for MIPA and proper pexophagosome formation		✓	✓
<i>PEP4</i>	Vacuolar protease			✓
<i>PEX3</i>	PMP peroxin required for peroxisome biogenesis and for recruitment of pexophagy receptor	✓	✓	✓
<i>PEX14</i>	PMP peroxin required for peroxisome biogenesis and for recruitment of pexophagy receptor	✓	✓	✓
<i>PIK1</i>	PtdIns-4-kinase required for MIPA formation			✓
<i>PFK1</i>	Subunit of phosphofructokinase complex			✓
<i>TUP1</i>	Transcriptional repressor	✓		

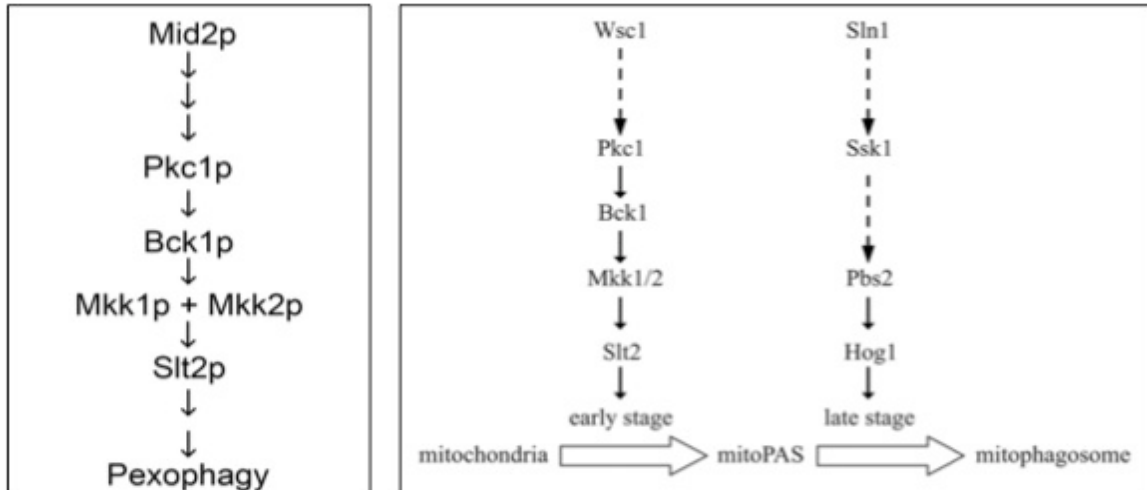


Figure 1-2: Schematic of proposed MAPK involvement in pexophagy and mitophagy [34][35]. Slt2 is required for pexophagy and involved in mitophagy. The direct target(s) of Slt2 in this process remains unknown.

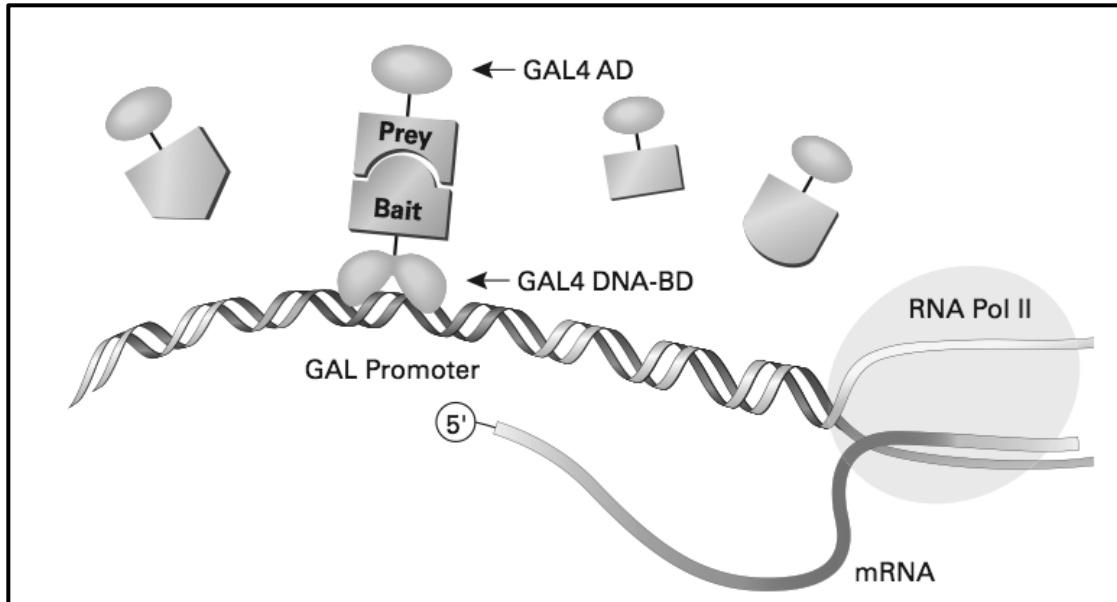


Figure 1-3: Yeast two-hybrid schematic. The yeast two-hybrid assay can be used to identify protein-protein interactions via an artificial system in which the two relevant proteins are expressed separately, one (the “bait” protein) fused to the Gal4 DNA-Binding Domain (BD) and one (the “fish” protein) fused to the Gal4 transcriptional activation domain (AD). In the specially-designed background strain, activation of the reporter genes (*HIS3* and *ADE2*) only occurs when the “bait” and “fish” proteins interact and bind to the Gal4-sensitive promoters. Strains with various combinations of bait and fish proteins expressed are grown on media deficient in Histidine or Adenine to determine which pairs of proteins interact.

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II. Results

Atg30 and Atg36 contain AIMs and phospho-regulated sites that are required for Atg8 and Atg11 interaction

While Atg36 (the *S. cerevisiae* pexophagy receptor) and PpAtg30 do not share sequence homology, they are functionally orthologous and interact with Pex3 of the peroxisomal membrane and Atg8 and Atg11 of the autophagy machinery (Motley *et al.*, 2012; Farré *et al.*, 2013). However, a functional AIM and the specific phospho-sites required for Atg8 and Atg11 interaction remained to be described for both pexophagy receptors.

In order to investigate which residues on Atg30 and Atg36 may be involved in Atg8 interaction, we employed a yeast two-hybrid approach. Wild-type Atg30 did not show interaction with Atg8 in this assay. The interaction between the mammalian optineurin receptor (OPTN) and LC3 (an Atg8 homolog) is enhanced upon phosphorylation at residues near its LIR (Wild *et al.*, 2011). Therefore, it is possible that proper activation by phosphorylation of *P. pastoris* Atg30, which might not occur in the yeast two-hybrid system because it is performed in a heterologous yeast (*S. cerevisiae*), is preventing its interaction with Atg8. To prove this hypothesis, we mutated a residue of Atg30 just upstream of a putative AIM by replacing it with either a phosphomimic amino acid (Glutamate) or an amino acid that cannot be phosphorylated (Alanine). When Serine-71 of Atg30 was mutated to Glutamate, Atg30^{S71E}-Atg8 interaction was observed. This residue is just upstream of the Atg30 site, WILF (W73-F76). Interestingly, when this AIM site was mutated along with Serine-71 (Atg30^{S71E,W73A, F76A}), Atg30-Atg8 interaction did not occur, confirming that WILF sequence was the real AIM site for Atg30.

To extend this study to *S. cerevisiae*, we used consensus sequence analysis to screen for a putative AIM in ScAtg36 with an upstream Serine residue that shared some homology with PpAtg30. In order for ScAtg8 interactions to be observed in these experiments, the C-terminal residues Glycine-116 and Arginine-117 were first removed via site-directed mutagenesis to form a non-conjugatable mutant, Atg8^{F115}. We then used the two-hybrid system to determine which of these potential sequences, if any, were required for Atg8-Atg36 interaction. Unlike the *P. pastoris* proteins, ScAtg8 interaction with wild-type ScAtg36 is observed via yeast two-hybrid, most probably because the interaction study was performed in *S. cerevisiae*. In the N-terminal region of ScAtg36, we identified a putative classical WxxL-like sequence, FEVL (F33-L36). Mutation of both Phenylalanine-33 and Leucine-36 to Alanine (Atg36^{F33A, L36A}) abolishes the Atg8-Atg36 interaction. Interestingly, mutation, of Serine-31 to Alanine (Atg36^{S31A}), upstream of the AIM prevents Atg8-Atg36 interaction.

Based on comparison of the sequence organization of Atg30 and Atg36, we predicted that Atg36 would also have an Atg11 binding site following the AIM. It was previously shown that Serine-112 of Atg30 is required for Atg30-Atg11 interaction (Farré *et al.*, 2008). Here we show that Atg11 interacts with wild-type Atg36. However, mutation of Serine-97 to Alanine causes loss of the Atg11-Atg36 interaction. In addition, we wanted to investigate whether mutation of the putative AIM site (F33-L36) or Serine-31 of Atg36 would affect Atg36 binding to Atg11. Not surprisingly, Atg11 is capable of interacting with Atg36 mutants (Atg36^{F33AL36A} and Atg36^{S31A}) incapable of Atg8 interaction.

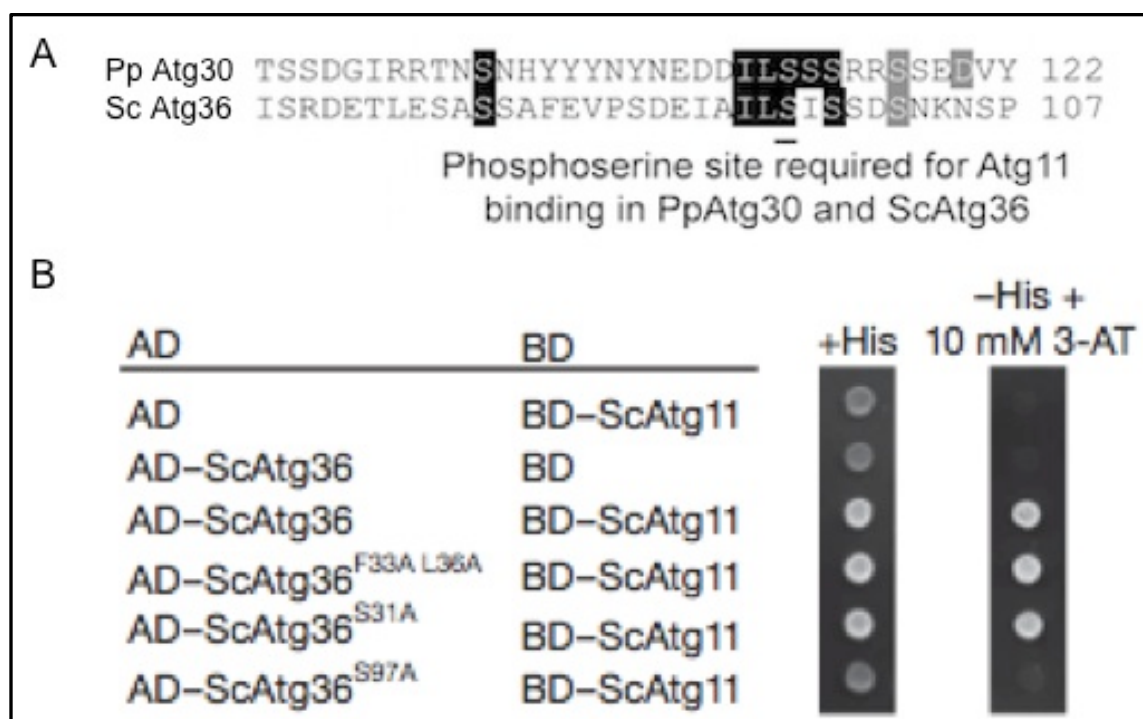


Figure 3: Yeast two-hybrid analysis of ScAtg36-Atg11 interaction [2]. A. Sequence alignment of PpAtg30 and ScAtg36 in the region containing Atg11 binding sites. B. AH109 cells were transformed with pairs of yeast two-hybrid assay plasmids (indicated by “AD” and “BD” columns). AD plasmids encode wild-type or mutant forms of ScAtg36 as indicated. BD plasmids encode wild-type Atg11. Empty vectors were used as negative interaction controls and strains were grown on +His and -His +10 mM 3-AT plates.

Atg8 and Atg11 interact with Atg36 independently and non-simultaneously

Although the specific Atg8 and Atg11 binding sites for both Atg32 and Atg36 had been confirmed, the specific manner in which receptor proteins bind to the machinery proteins is yet to be determined. Interestingly, we found that these two binding sites were always in close proximity, and in some yeast, they were overlapping, which might suggest that Atg8 and Atg11 cannot interact simultaneously with the receptor. In order to investigate the interaction mechanism, we employed a yeast three-hybrid system.

The yeast three-hybrid system utilizes pBridge, a plasmid that encodes both a Gal4 DNA Binding domain fusion protein and a protein fused to a nuclear localization signal (NLS) (Mitchell *et al.*, 2008). This assay can help determine whether two proteins (in this case Atg8 and Atg11) can compete for interaction with another protein (Atg36). In one experiment, ScAtg11 was fused to a nuclear localization signal (NLS-ScAtg11). NLS-Atg11 was able to disrupt the interaction between BD-ScAtg8 and wild-type ScAtg36. When the Atg11-binding site of ScAtg36 (Serine-97) was mutated to Alanine (ScAtg36^{S97A}), NLS-Atg11 could not compete with Atg8 for Atg36 interaction. In order to determine if Atg8 could also compete with Atg11 for interaction, Atg8 was then fused to an NLS in the pBridge plasmid expressing BD-Atg11. Surprisingly, NLS-Atg8 could not disrupt the interaction between interaction between Atg11 and wild-type Atg36.

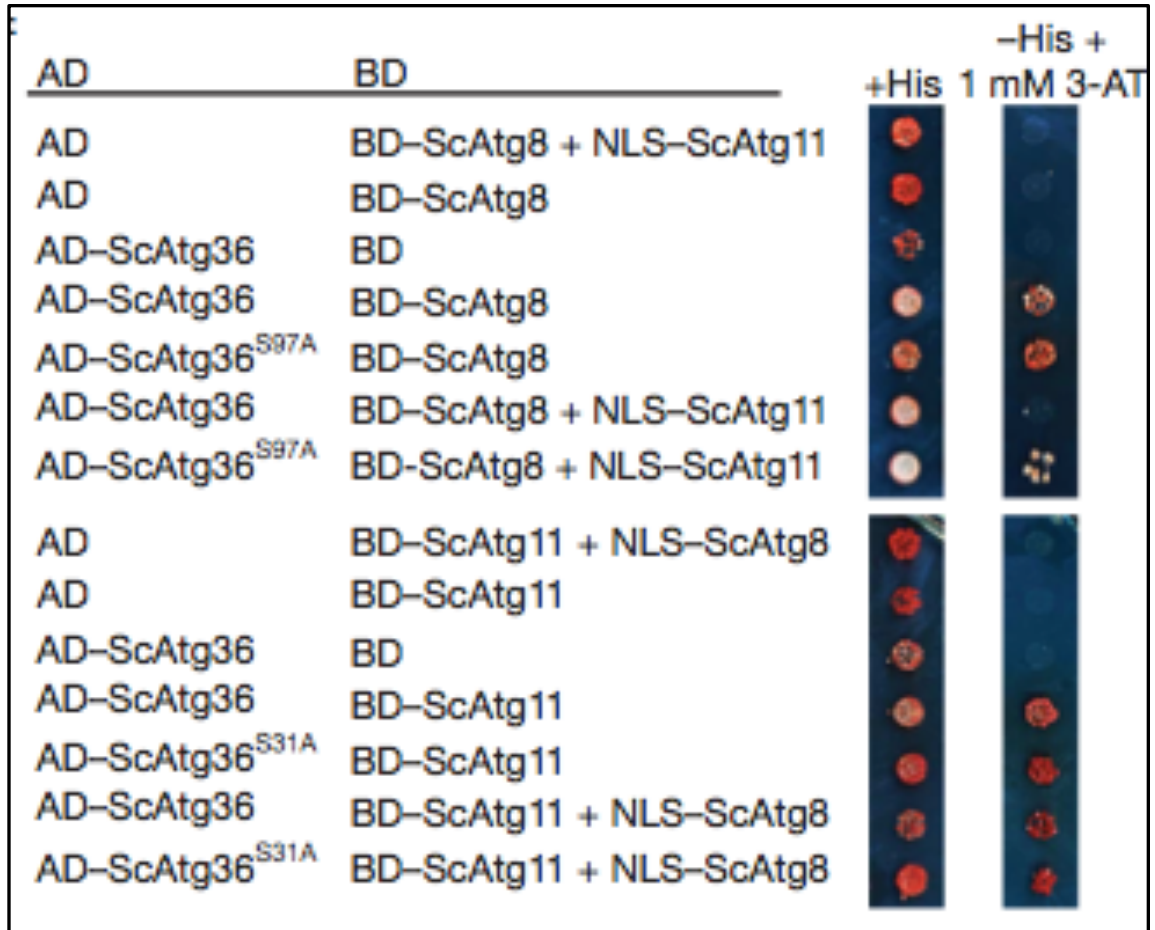


Figure 4: Competition-based yeast 3-Hybrid Analysis of Atg36 with Atg8 and Atg11 [2]. HF7c cells were transformed with pairs of yeast two-hybrid plasmids (indicated by “AD” and “BD” columns). The yeast three-hybrid assay is an extension of the yeast two-hybrid system, utilizing the co-expression of a third protein as a competitor as indicated in the figure (NLS-ScAtg11 or NLS-ScAtg8) in the pBridge-BD vector. ScAtg36 mutants affected in either Atg11 binding (ScAtg36^{S97A}) or in Atg8 binding (ScAtg36^{S31A}) were included where indicated, as were appropriate auto-activation and interaction controls. Strains were grown on +His and -His +1 mM 3-AT plates.

The effect of phospho-regulation of Atg36 on pexophagy in vivo

It is known that Atg36 interacts with both Atg8 and Atg11 by yeast two-hybrid and *in vivo* as shown in this and other work (Motley *et al.*, 2012). To extend our investigation of these phospho-regulated sites, we assessed their effects on the pexophagy pathway using *in vivo* studies. We followed the level of pexophagy in various strains in a wild-type background by assaying the appearance of free GFP over time caused by the degradation of the peroxisomal matrix marker, Thiolase-GFP, under pexophagy-inducing conditions. Not surprisingly, the $\Delta atg36$ strain exhibited a full block in pexophagy comparable to the $\Delta atg1$ mutant used as a negative control for pexophagy because Atg1 is required for all autophagy-related pathways).

When the $\Delta atg36$ strain (in BY4742 background) was complemented with a vector expressing wild-type Atg36 fused to an HA-tag (HA-ScAtg36), pexophagy was restored to levels observed in the wild-type strain BY4742. Interestingly, the expression of HA-ScAtg36^{F33AL36A} (the AIM mutant) in the $\Delta atg36$ background caused a delay in pexophagy, similarly to the expression of Atg36^{S31A} (Atg8 phospho-site mutant). Furthermore, the expression of the Atg36^{S97A} point mutant in the $\Delta atg36$ background caused a pronounced pexophagy defect comparable to that seen in the Atg11 binding site mutant of Atg30 (Farré *et al.*, 2008).

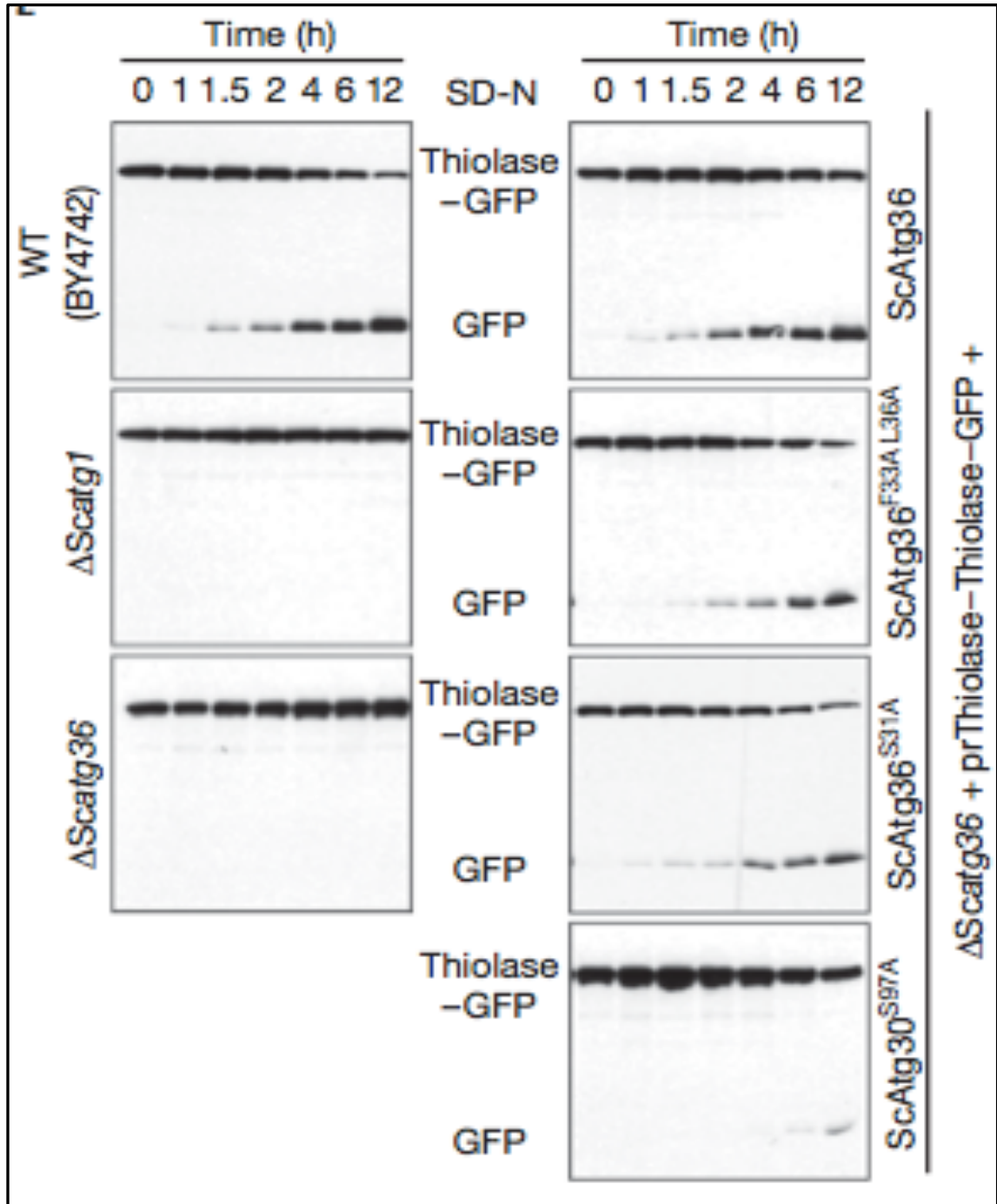


Figure 5: Assay monitoring pexophagy in wild-type and mutant *S. cerevisiae* cells expressing Thiolase-GFP [2]. Cells were cultured in oleate medium until mid-log growth and then shifted to SD-N. Pexophagy was monitored by immunoblotting with -GFP antibodies. WT and $\Delta atg1$ cells served as positive and negative controls for normal levels of pexophagy. $\Delta atg36$ cells expressing either HA-Atg36 WT, HA-Atg36^{F33A, L36A}, HA-Atg36^{S31A}, or HA-Atg36^{S97A} as indicated in the figure were cultured and monitored for pexophagy.

Phospho-regulated sites of Atg32 required for Atg8 and Atg11 interaction

Atg32 contains a WQAI motif near its N-terminus that is required for Atg8-Atg32 interaction (Okamoto *et al.*, 2009b). However, the presence of phospho-regulated sites that mediate Atg32 interaction with Atg8 had not been confirmed.

We performed a yeast two-hybrid experiment to analyze the interactions of Atg32 with Atg8 and Atg11 and determine which specific sites are required for these interactions. GBT9 plasmids were used to encode either Atg8^{F115} or Atg11 fused to the Gal4 DNA Binding Domain. PGAD-GH plasmids were used to encode wild-type and mutant forms of Atg32 fused to the Gal4 Activation Domain. The three Serine residues upstream of the Atg32 AIM, Serine-81, Serine-83, and Serine-85, were mutated to Alanine (Atg32^{S81A, S83A, S85A}). While BD-Atg8 interacts strongly with wild-type AD-Atg32, it was unable to interact with AD-Atg32^{S81A, S83A, S85A}. It was also demonstrated that BD-Atg8 can interact with Atg32^{S114A, S119A}, in which the Atg11 binding site was mutated.

As mentioned in a previous study, Serine-114 and Serine-119 must be phosphorylated for interaction with Atg11 to occur. We showed via two-hybrid that Atg11 can interact with wild-type AD-Atg32. Not surprisingly, Atg11 did not interact with AD-Atg32^{S114A, S119A}. However, Atg11 interaction with AD-Atg32^{S81A, S83A, S85A} was shown.

AD	BD	+His	-His + 10mM3-AT
AD	BD-ScAtg8		
AD-ScAtg32	BD		
AD-ScAtg32	BD-ScAtg8		
AD-ScAtg32 ^{S81A S83A S85A}	BD-ScAtg8		
AD-ScAtg32 ^{S114A S119A}	BD-ScAtg8		
AD	BD-ScAtg11		
AD-ScAtg32	BD		
AD-ScAtg32	BD-ScAtg11		
AD-ScAtg32 ^{S81A S83A S85A}	BD-ScAtg11		
AD-ScAtg32 ^{S114A S119A}	BD-ScAtg11		

Figure 6: Yeast two-hybrid analysis of ScAtg32 interactions [2]. AH109 cells were transformed with pairs of yeast two-hybrid assay plasmids (indicated by “AD” and “BD” columns). AD plasmids encode Gal4-AD fused to the indicated wild-type or mutant forms of Atg32. Atg32 was mutated at Serine-81, 83 and 85 (upstream of the AIM [9]) and Serine-114 and 119 Atg11 binding site). BD plasmids encoded Gal4 DNA-BD fused to wild-type Atg8 or Atg11 as indicated. Empty vectors were used as negative interaction controls and strains were grown on +His and -His +10 mM 3-AT plates.

The role of Slt2/MAPKs in pexophagy/mitophagy and regulation of selective autophagy

While several mechanistic details of selective autophagy have been determined by this work and previous studies, little is known about the signaling pathways that link environmental and intracellular stimuli and conditions to activation of pexophagy and mitophagy. Perhaps one of the most important steps to take in developing insights on selective autophagy signaling is to identify the kinase that phosphorylates the Atg8 and Atg11 binding sites of Atg30, Atg32, and Atg36. In order to accomplish this, we must identify candidate kinases that affect specific selective autophagy pathways and not other autophagy-related pathways. One such kinase, Slt2, is a MAP Kinase that has been shown to cause a defect in pexophagy and mitophagy *in vivo* but not other selective and nonselective forms of autophagy (Manjithaya *et al.*, 2010; Mao *et al.*, 2011). We first chose to investigate the effects of Slt2 on the interactions of Atg32 and Atg36 with the Atg8 and Atg11 via yeast two-hybrid.

In order to investigate these effects, a yeast two-hybrid assay was performed in a strain in which the *SLT2* gene was deleted. Remarkably, interactions of ScAtg32 and ScAtg36, with both Atg11 and Atg8 were lost in the $\Delta slt2$ background (Figure 6 and 7). To confirm that it was the explicit removal of Slt2 causing this loss of interaction, Slt2 fused to a nuclear localization signal (NLS-Slt2) was reintroduced in the $\Delta slt2$ strain. The interaction of Atg32 with Atg8 was restored with NLS-Slt2 expression (Figure 8).

In order to determine if Slt2 affected phosphorylation of other selective autophagy receptors, we again employed a yeast two-hybrid assay studying the interactions of Atg36 with Atg8 and Atg11. In this assay, we used the wild-type strain (AH109) and $\Delta atg1$ to

compare the relevant Atg36 interactions with those in $\Delta slt2$. In the wild-type strain, Atg36-Atg8 interaction occurred normally, in addition to Atg8 interaction with Atg19, the Cvt pathway receptor. These interactions were also observed in $\Delta atg1$. Interaction of Atg11 with Atg36 as well as Atg19 was also observed in both the wild-type strain and $\Delta atg1$ (Figures 9 and 10). These results show that Atg1 kinase is not regulating these binding events.

As shown previously, the $\Delta slt2$ strain was defective for both Atg36-Atg8 interaction as well as Atg36-Atg11 interaction. Interestingly, the interactions of Atg19 (the Cvt receptor protein) with Atg8 and Atg11 were not affected by the absence of Slt2 (Figures 9 and 10). In addition, the interactions of Atg36 with both Atg8 and Atg11, in the $\Delta slt2$ strain, were rescued by introducing the phosphomimic mutations at the Atg8 binding site (Atg36^{S31E}) and the Atg11 binding site (Atg36^{S97E}). In the $\Delta slt2$ strain, addition of NLS-Slt2 by co-expression in the pBridge plasmid also restored both the Atg36-Atg8 and Atg36-Atg11 interactions.

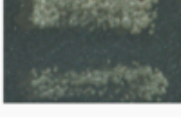
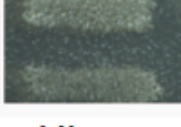
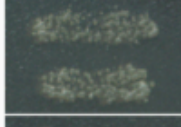
AD	BD	+His	-His + 10mM 3AT
ScAtg32 WT	ScAtg11 WT		
AD	ScAtg11 WT		
ScAtg32 WT	BD		
PpAtg30 WT	PpPex3 WT		
AD	BD	+His	-His + 10mM 3AT
ScAtg32 WT	ScAtg8 WT		
AD	ScAtg8 WT		
ScAtg32 WT	BD		
PpAtg30 WT	PpPex3 WT		

Figure 7: Yeast two-hybrid interaction studies of Atg32 in $\Delta slt2$ cells [3]. AH109 $\Delta slt2$ cells were transformed with pairs of yeast two-hybrid assay plasmids (indicated by “AD” and “BD” columns). AD plasmids encode Gal4-AD fused to wild-type Atg32. BD plasmids encoded Gal4 DNA-BD fused to either wild-type Atg8 or Atg11 as indicated. Empty vectors were used as negative interaction controls and PpAtg30 x PpPex3 was used as a positive interaction control. Strains were grown on +His and -His +10 mM 3-AT plates.

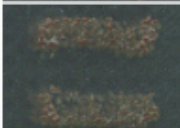
AD	BD	+His	-His + 10mM 3AT
ScAtg36 WT	ScAtg11 WT		
AD	ScAtg11 WT		
ScAtg36 WT	BD		
PpAtg30 WT	PpPex3 WT		
AD	BD	+His	-His + 10mM 3AT
ScAtg36 WT	ScAtg8 WT		
AD	ScAtg8 WT		
ScAtg36 WT	BD		
PpAtg30 WT	PpPex3 WT		

Figure 8: Yeast two-hybrid interaction studies of Atg36 in $\Delta slt2$ cells. AH109 $\Delta slt2$ cells were transformed with pairs of yeast two-hybrid assay plasmids (indicated by “AD” and “BD” columns). AD plasmids encode Gal4-AD fused to wild-type Atg36. BD plasmids encoded Gal4 DNA-BD fused to either wild-type Atg8 or Atg11 as indicated. Empty vectors were used as negative interaction controls and the combination of PpAtg30 x PpPex3 was used as a positive interaction control. Strains were grown on +His and -His +10 mM 3-AT plates.

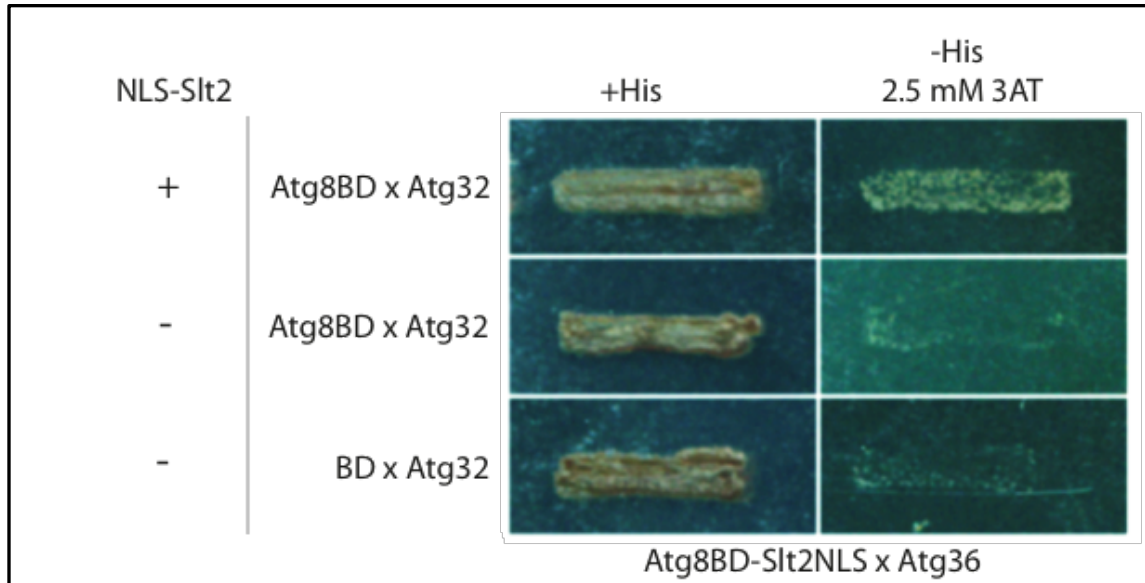


Figure 9: NLS-Slt2 co-expression restores Atg8-Atg32 interaction in Δ *slt2* cells. The Δ *slt2* strain was transformed with various pairs of BD and AD plasmids, encoding wild-type Atg8 and Atg32 respectively. An empty BD vector was used as a negative control. The pBridge plasmid with NLS-Slt2 co-expression was included where indicated (with “+”).

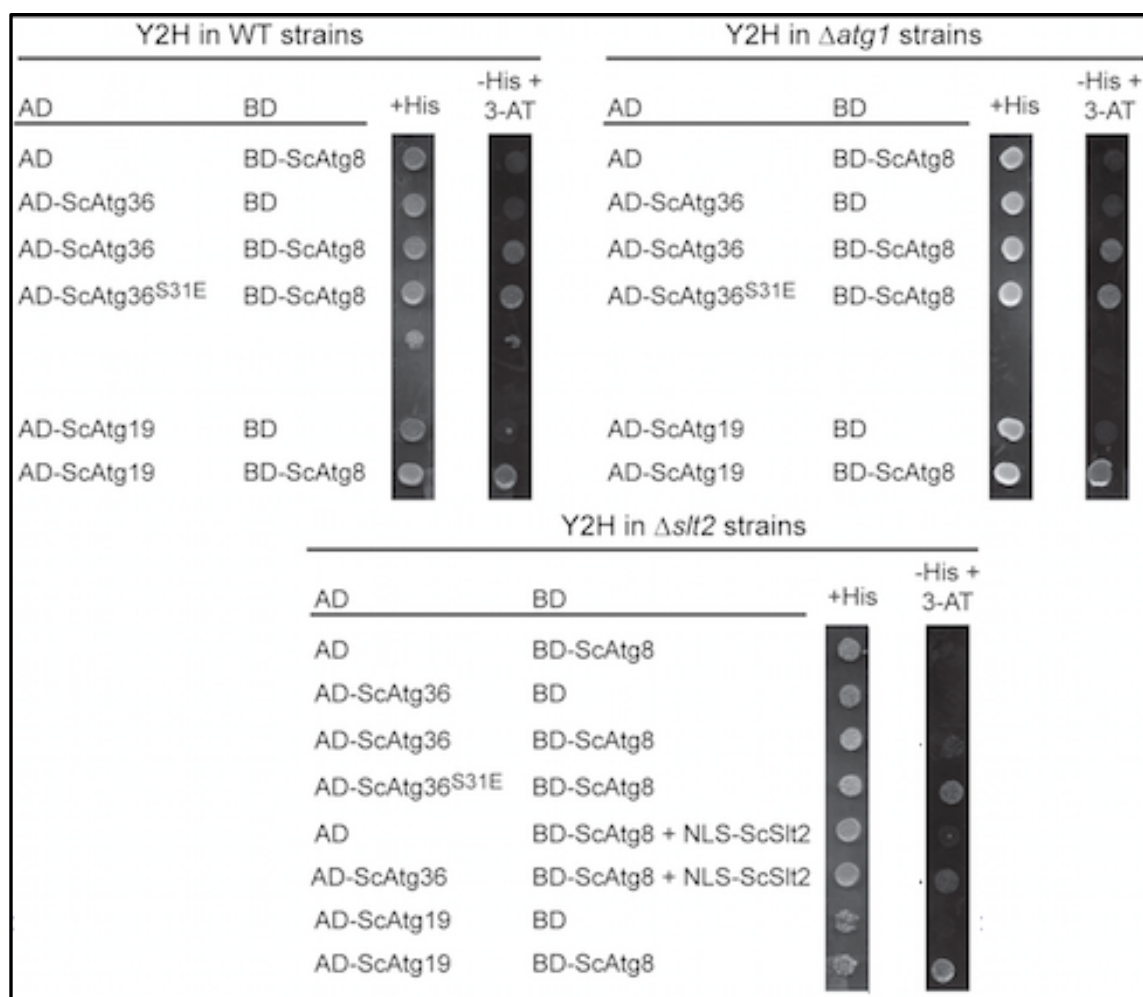


Figure 10: Yeast two-hybrid analysis of Atg36-Atg8 interactions in *S. cerevisiae* wild-type, $\Delta atg1$, and $\Delta slt2$ strains. In this assay, wild-type, $\Delta atg1$, and $\Delta slt2$ strains were used. Both wild-type (“AD-Atg36”) and Atg8 binding site phosphomimic mutant (AD-Atg36^{S31E}) forms of Atg36 were used. In the $\Delta slt2$ strain, the pBridge plasmid was used to co-express NLS-Slt2 along with BD-Atg8 where indicated in the figure. The Atg19-Atg8 interaction was also studied in this assay, and all appropriate auto-activation controls were included. Empty vectors were used as negative interaction controls and strains were grown on +His and -His +1 mM 3-AT plates.

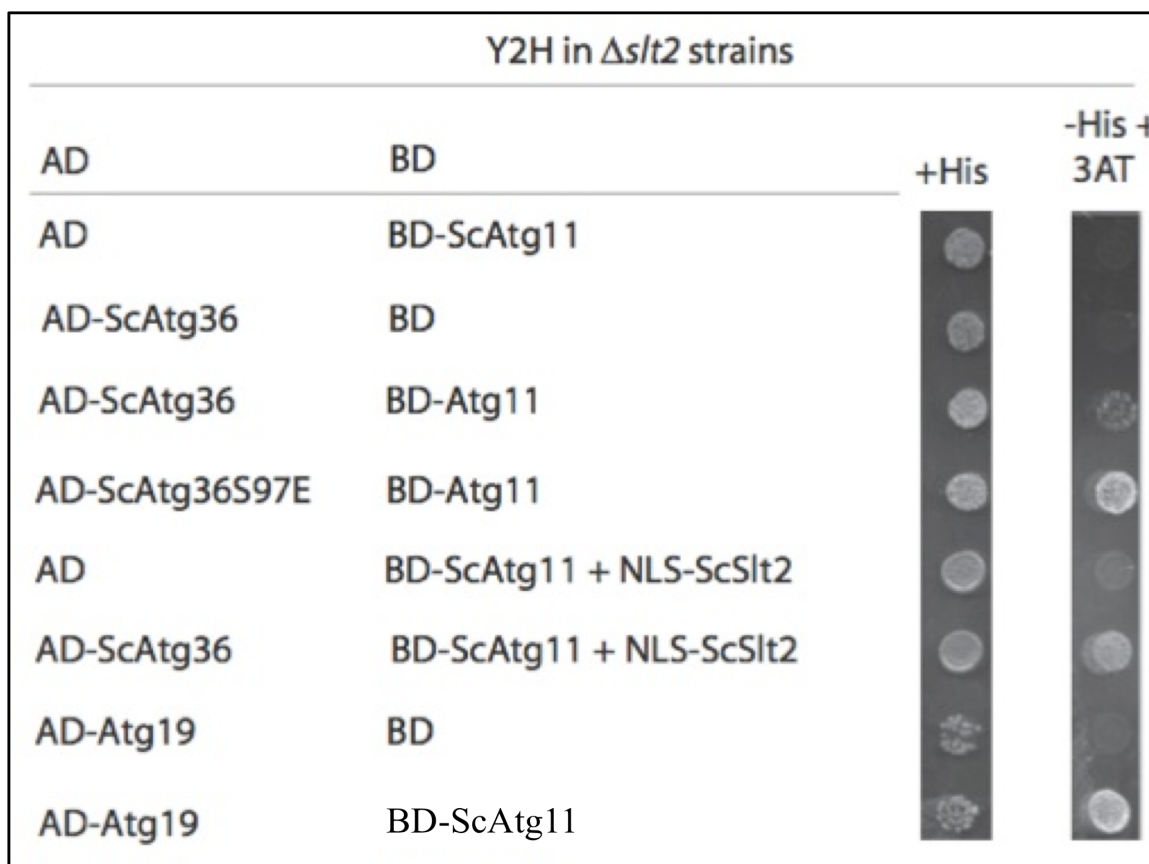


Figure 11: Yeast two-hybrid analysis of Atg36-Atg11 interactions in the *S. cerevisiae* $\Delta slt2$ strain. This assay was used to further analyze the role of Slc2 in the Atg36-Atg11 interaction. Both wild-type (“AD-Atg36”) and Atg11 binding site phosphomimic mutant (AD-Atg36^{S97E}) forms of Atg36 were used. The pBridge plasmid was also used to co-express NLS-Slc2 along with BD-Atg11 where indicated in the figure. The Atg19-Atg8 interaction was also studied in this assay, and all appropriate auto-activation controls were included. Empty vectors were used as negative interaction controls and strains were grown on +His and -His +1 mM 3-AT plates.

The role of Kinase(s) in regulation of the Pichia pastoris pexophagy receptor Atg30

As mentioned, interaction between wild-type PpAtg30 and PpAtg8 via yeast two-hybrid was not previously observed without manipulation of PpAtg30 via mutagenesis (Figure 1). In order to further investigate the role of kinases in phosphorylation of the *P. pastoris* pexophagy receptor, we co-expressed various candidate kinases along with the two-hybrid fusion proteins. Based on our findings in *S. cerevisiae*, we used the NCBI BLAST Alignment Tool to identify *P. pastoris* proteins that shared close homology with ScSlt2. This search yielded two results for proteins in the *P. pastoris* genome, here referred to as “Slt2-like protein” (a protein that shared the closest homology with ScSlt2) and “Smk1-like protein” (one that was closely homologous to Smk1 in *S. cerevisiae*). We adapted the yeast three-hybrid system to investigate this by co-expressing these candidate kinases fused to a nuclear localization signal (Figure 11). When the Slt2-like *P. pastoris* protein was co-expressed with PpAtg8 in the pBridge plasmid, Atg8-Atg30 interaction was not observed. In addition, when the Smk1-like *P. pastoris* protein was expressed, Atg8-Atg30 interaction was again not observed.

Sequence analysis of the pexophagy and mitophagy receptors in *P. pastoris* and *S. cerevisiae* reveals that they all contain many regions with Serine and Threonine residues that can be phosphorylated. These residues are often found within sites that share homology with consensus sequences.

When PpHrr25, a Casein Kinase I-like protein was expressed, Atg8-Atg30 interaction did occur via yeast two-hybrid. Interestingly, Hrr25 co-expression did not promote Atg8-Atg32 interaction in this system, showing that Hrr25 regulates a pexophagy-specific interaction between Atg8 and Atg30.

PpAtg30
MFSRKQVQKRNNELSSLHC SNSS NSLNRIHKNEETAKGTVGVNARGNNRSDNVA SPGOLR
PRTSS ILTDNSEWILF SPENAEGEYVI TSSD GIRRTNSNHYYNYNE DILSSSRSSSED
VYDAEQEYTEQPVNNHVQVEDEEDDDSI INDLTHVDDYDYEEDDKQDLTTRIDNWRKK
QVSELLNELNHDDDLDPVLNRDKIDLIQSWGIENEKLNTPRAKKRQRKSKRASFGQDL
LSKYSMEDLKI IKQIVAQLRDDLDKVKHDKPS SPLPNYHNTLKQAP SSNSQNPSFIS YYS
NYLTKNN SQOTFNSQSTSGS LLNPNPLEKYLPLFLKNLLYED SNGSHQHPET SEKEHFW
NDLKS VNSSILT LSSNS KLKQEIL

PpAtg32
MKQTYYPKVAEPIGSE SMDDSKLD DMVQSLPVDPLLD TPFFRSADVEQGDTFATENN
AGPTO SARTNVNFLPPHQ SLFDALQSSNLNMVEEIDPLRRSNOPEEC SPSCWNQEF SVTA
WQMVHRDDVKLQNLWTHTKMYGTGSSRRVPGTGNG SVLSSEYEETDLPDTP LLNEDDIE
ERNEHDTILMIAEDENLKDTATVSSGRQSSFVMPKV SLPDSGASNSKSI SWNGKHARIKV
QVVGNCARLLARLIPYRKT LWMLDFVE SGAD ILVLLLNKDNVVLPRILNKPFLPI IKD
DDAEFSS SIDTTS LFDNYNTKSTAR SSSSSSI ISEPLELKS IDDDLFDI IHAL SNMELDFC
GEESFQHI STQTSTIAS SKQARSRRSSSRLAQRRLHKQTQMSQSREKRSHQRC SYEYIN
WIRRLTLGLTLGLISISYCIW SPSS DHNKLHGYHMHMGVWMRCTV

ScAtg30
MASVNNYQVDCGSR SARIQPRINNGIHDEE SIFVLEL SEEFELDFHRLK SFNDVRVIN
NPDLS PECTNTAISRD ETLESASSAFEVPSDEIAI LSISSDSNKN SPSEQPAPALRNIR
SSNSD RIDEWCLGSHLFNELHQNVPSQSSDGVNHGFPVYS PKERELYTS AKLKKLTNAQR
IAVQKLSRDLYPILRTC YREKTRRQLLTYHHERIFDDI PSFFPQORDFI FNYYSMPLFDR
LSDVD IDS SRSRFTDESTGETLNR SPAASSSLENTSWFGWTLLSRPLDREW

ScAtg32
MVLEYQOREGKGS SSKSMPPD SSSTTIHTCSEAQTGED KGLLDPHLSVLELLSKTGHS SPS
PMGQNLVTSIDISGNHVNDSISGSWQAI QPLDLGASFIPERC SSQTTN SILSSSDTSE
EEQELLQAPAADI INI IKQGQEGANVVSF SHPFKQLQKI ISLPLPGKEK TPFNEQDDGD
EDEAFEEDSVTITK SLTSSSTNS FVMPKLSLTQKNPVFRLILGR TGSSFYQSIPKEYQSL
FELPKYHDSATFPQYTGIV IIFQELREMVSLNRIVQYSQGKVPVIPCQPGQVIQVKNVL
KSFLRNKLVKLLFPFVVVTNKRDLKKMFQRLQDLSLEYGEDVNEDNDDEAIHTKSRSYC
RNKKAENSKKK SPKSNKKPKRKKQKFFT SWFTWGISITIGISFGCCVTYFVTAAYEHQTV
KSLSLRPSILASLLSLDSSSDTINTPATAS SSTEQFLWFDKGT LQINFHSDGPFIMKSLT
IIKETWGMNTFVLHALSKPLKFLNENLKS SEFSIDE SNRILALGYILL

Figure 12: Putative phosphorylation sites in sequences of *P. pastoris* and *S. cerevisiae* pexophagy and mitophagy receptors. Orange boxes denote the AIMs of each protein sequence (with Atg8 binding sites upstream). Red boxes indicate locations of Atg11 binding site motifs. Teal highlighting indicates areas of sequence that are potential phosphorylation sites for Casein Kinase I and Casein Kinase 2[3][4]. Yellow highlighting indicates areas of sequence that are potential phosphorylation sites for MAP Kinases.

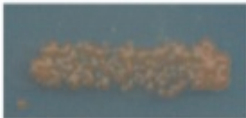
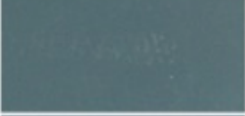
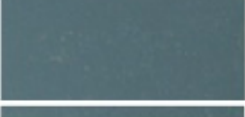
AD	BD	+His	-His +2.5mM 3AT
PpAtg30	Atg8BD+NLSSlt2		
PpAtg30	BD+NLSSlt2		
PpAtg30	Atg8BD+NLSSmk1		
PpAtg30	BD+NLSSmk1		
PpAtg30	Atg8BD+NLSHrr25		
PpAtg30	BD+NLSHrr25		

Figure 13: Yeast two-hybrid interaction studies of PpAtg30 with PpAtg8 and co-expression of various *P. pastoris* kinases. In this experiment, PpAtg8-PpAtg30 interactions were studied with co-expression of a PpSlt2-like protein, PpSmk1-like protein, and PpHrr25 using the pBridge vector in an *S. cerevisiae* background. Appropriate auto-activation controls for the AD-PpAtg30, AD-PpAtg32, and BD-PpAtg8 + NLS-PpHrr25 plasmids were included as shown.

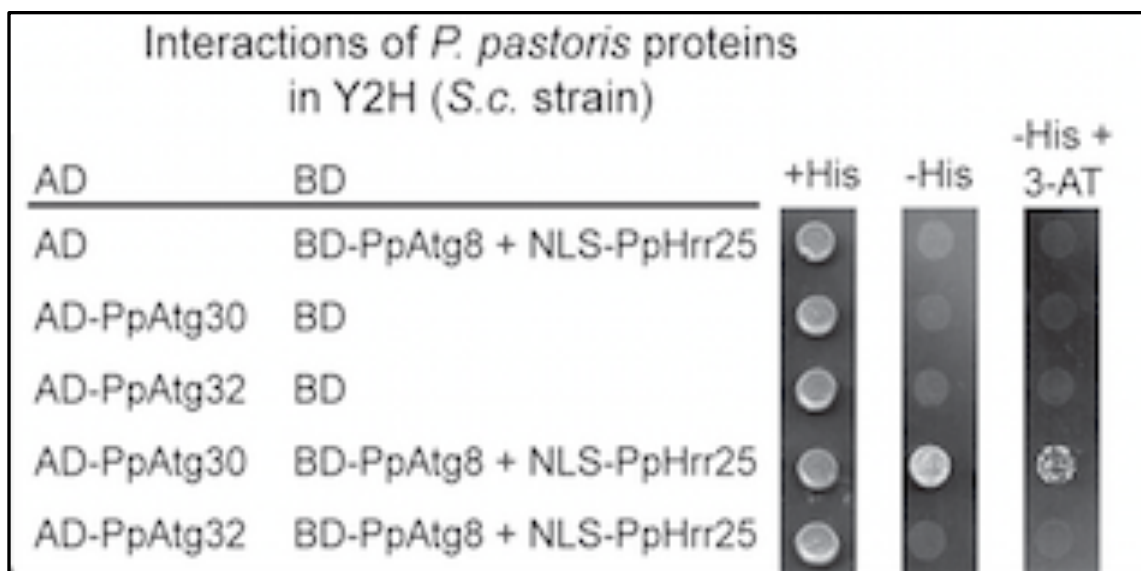


Figure 14: Yeast two-hybrid interaction studies of *P. pastoris* receptors with PpAtg8 and PpHrr25 co-expression. In this experiment, PpAtg8 interactions with PpAtg30 and PpAtg32 were studied with co-expression of PpHrr25 using the pBridge vector in an *S. cerevisiae* background. Appropriate auto-activation controls for the AD-PpAtg30, AD-PpAtg32, and BD-PpAtg8 + NLS-PpHrr25 plasmids were included as shown.

Acknowledgments

I would like to especially thank Dr. Jean-Claude Farré for providing many of the plasmids and strains used in these studies, assisting with the planning, execution, and analysis of all experiments, and for performing the sequence analyses shown in this work. I also thank Sarah Burnett for performing the pexophagy assay experiment shown in Figure 4 and assisting me with technical aspects of several of the yeast two- and three-hybrid experiments shown in this work. I also thank Dr. Jing-Jing Liu for the $\Delta atg36$ mutant strain used in the pexophagy assay experiment. I thank Dr. Subramani and Dr. Farré for helping me edit the written portions of this thesis.

The Results section, in part, contains data and findings that have been published in a 2013 EMBO Reports article titled “Phosphorylation of pexophagy and mitophagy receptors coordinates their interaction with Atg8 and Atg11” [*EMBO Rep.* 14(5): 441-9]. The corresponding author Dr. Suresh Subramani and co-authors Dr. Jean-Claude Farré and Sarah Burnett have approved the inclusion of these findings in this work.

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III. Discussion

Yeast Selective Autophagy Receptors contain AIMs and Atg11 binding sites that are regulated by phosphorylation

In this work, we have identified several specific sites of various yeast selective autophagy receptors that are essential for their proper functioning in their respective selective autophagy pathways. Identification of these sites, along with further analysis of the mechanism by which these receptors interact with Atg8 and Atg11 of the core autophagy machinery, has contributed to the establishment of a common model for phosphoregulation of selective autophagy receptors.

As shown for other selective autophagy receptors, we sought to identify the true AIMs of PpAtg30 and ScAtg36. We predicted that these AIMs would contain similar WxxL-like motifs. Although Atg30 and Atg36 do not show sequence homology, we demonstrated through sequence analysis that they contain similar motif organization. This sequence comparison allowed us to identify the true AIMs of each protein and demonstrate their direct interaction with Atg8 that has not been previously shown.

Due to the discovery that phosphorylation of optineurin (OPTN) just upstream of its AIM enhances LC3 interaction (Wild *et al.* 2011), we sought to find similar regulation for yeast pexophagy receptors. The study of *P. pastoris* protein interactions in the artificial yeast two-hybrid system presents a challenge, because these experiments must be done in a *S. cerevisiae* background. As a result, the proteins being studied may not be properly activated or modified in a manner that facilitates their true interactions *in vivo*. We predicted that mutation of a specific residue upstream of the true Atg30 AIM to a phosphomimic amino acid would facilitate Atg30-Atg8 interaction in this heterologous system. This interaction was also demonstrated *in vivo* by co-immunoprecipitation and

the phosphorylation of Atg30 at Serine-71 was confirmed by phosphopeptide mapping by mass spectrometry (Farré *et al.*, 2013). We found that Atg8 did in fact interact with Atg30^{S71E}. However, when Serine-71 was mutated along with this putative AIM site, the Atg30-Atg8 interaction was lost. This implies that the W73-F76 site, which does not strictly follow the AIM consensus W/F/YxxL/I/V, is the true AIM of Atg30. It is required for Atg8 interaction, and can be regulated by phosphorylation of an upstream Serine residue as was shown for OPTN in mammals.

An Atg8-interacting motif had not been previously identified in ScAtg36, although a previous study demonstrated Atg8 and Atg36 co-immunopurified in vivo, but they conclude it might be an indirect interaction (Motley *et al.*, 2012). By using in silico and yeast two-hybrid analysis, we identified this AIM, which is a canonical WxxL-like motif similar to that found for ScAtg32 and ScAtg19. When this site (F33-L36) was mutated, Atg8 could no longer interact. We further demonstrated this by showing that Serine-31, just upstream of the AIM, is also required for Atg8-Atg36 interaction. This finding suggests that this FEVL site of Atg36 is the site of its true AIM. Identifying these specific phospho-sites required for Atg8 interaction is a crucial step in elucidating the mechanisms that control pexophagy.

We extended these studies to investigate Atg11 interaction with the pexophagy receptors. Interestingly, we found that Atg11 could interact with Atg36 even when the AIM or Serine-31 was mutated to Alanine. However, Serine-97, when mutated to Alanine, prevented Atg36-Atg11 interaction. This Serine residue is located at a site that contains the conserved (D/S)ILSSS sequence found for other receptors. These findings also demonstrate that behavior for Atg36 that is conserved between selective autophagy

receptors in both *S. cerevisiae* and other yeast species. It also implies that Atg11 can interact with Atg36 independently from Atg8.

As shown for other selective autophagy receptors, we used an *in vivo* approach to confirm that these newly identified sites play an important role in pexophagy. We confirmed that these individual sites in Atg36 had significant phenotypic effects on the pexophagy pathway. Individual mutations to the AIM and Serine-31 only partially affected pexophagy. This result is consistent with the fact that both mutations similarly disrupt the Atg36-Atg8 interaction, and the finding that disruption of the Atg8-Atg30 interaction in *P. pastoris* also only partially delays pexophagy *in vivo* (Farré *et al.*, 2013). These findings demonstrate that Atg36 and Atg30 behave similarly in yeast two-hybrid analysis experiments as well as *in vivo* studies.

We predicted that Atg32 of *S. cerevisiae* would also contain specific sites that are regulated by phosphorylation. Atg32 contains a classical AIM, the site WQAI (W86-I89) that is required for Atg8 interaction (Okamoto *et al.*, 2009b). We discovered that at least one of the residues mutated in our two-hybrid analysis (Serine-81, Serine-83, and Serine-85) is phosphorylated to enhance this interaction. However, it is yet to be determined which site specifically, or which combination of these three sites, is phospho-regulated. We also confirmed by yeast two-hybrid that Serine-114 and Serine-119 are required for Atg11 interaction, as shown in a previous study (Aoki *et al.*, 2011). Further, we demonstrated that Atg8 could interact with the receptor protein when the Atg11 binding site is mutated, and vice versa. This finding demonstrates the evolutionary conservation of selective autophagy receptors in yeast that are characterized by their tripartite

interaction between the selected cargo, Atg8 and Atg11; some of these interactions are regulated by phosphorylation.

Mechanism of Interaction of ScAtg36 with Atg8 and Atg11

Some receptors found in nature contain overlapping Atg8 and Atg11 binding sites (Farré *et al.*, 2013) that may preclude their ability to interact with the two machinery proteins simultaneously. It is known that binding of both Atg8 and Atg11 to selective autophagy receptors is required for their proper functioning. Atg8 and Atg11 may have to interact with each receptor molecule separately and non-simultaneously. We chose to investigate this mechanism using *S. cerevisiae* Atg36, because phosphorylation of the receptor happens normally in the yeast two-hybrid system. Although we found that the Atg8 and Atg11 sites in ScAtg36 do not overlap, we wanted to determine if this model may be conserved among multiple selective autophagy receptors using a competition-based yeast three-hybrid analysis.

Surprisingly, we found that NLS-Atg11 can compete with Atg8 for Atg36 binding in this experiment. Therefore, if Atg8 binds first to Atg36, Atg11 is capable of disrupting this interaction. This competition was inhibited by mutation of Serine-97 to Alanine, which is consistent with our previous two-hybrid findings. However, we also showed that NLS-Atg8 is not capable of disrupting Atg11-Atg36 binding. This suggests that Atg11 may have a higher binding affinity for Atg36 than Atg8 does. It is possible that when Atg11 binds to Atg36 and other receptors, modulation of the respective binding affinities of Atg8 and Atg11 occurs to prevent interaction of Atg8 with the receptor. In order for Atg8 to displace Atg11, another regulatory event may need to occur, such as the

phosphorylation of the Atg8 site or dephosphorylation of the Atg11 site of Atg36. While it is still unknown which kinase(s) or phosphatase(s) perform these regulatory events, these findings shed new light on the mechanisms by which selective autophagy receptors interact with Atg8 and Atg11.

Implication of Kinases in Regulation of Selective Autophagy Receptors

The MAPK Slt2 has been implicated in both pexophagy and mitophagy, but not other selective autophagy pathways. Based on these findings, we predicted that Slt2 might play a role in the phosphorylation of pexophagy and mitophagy receptors. Our yeast two-hybrid results show that in *S. cerevisiae*, deletion of SLT2 disrupts interaction between wild-type Atg32 and Atg8 and Atg11 (Figure 6). We also show via two-hybrid that interaction of wild-type Atg36 with Atg8 and Atg11 is lost in the $\Delta slt2$ strain (Figure 7).

Introduction of a phosphomimic mutation at the Serine-31 and Serine-97 sites of Atg36 caused restoration of the interactions with Atg8 and Atg11 respectively. Our finding that deletion of SLT2 does not affect the interactions of Atg19 of the Cvt pathway imply that Slt2 is specifically involved in pathways that regulate pexophagy and mitophagy, but not other autophagy pathways. These discoveries are consistent with previous implications of Slt2 in both pexophagy and mitophagy and not the general autophagy or Cvt pathways. Our findings suggest that Slt2 plays an essential role in the signaling events that connect environmental and/or intracellular signals to the activation of pexophagy and mitophagy. It is clear that Slt2 is involved in the pathway that results in phosphorylation of ScAtg32 and Atg36 at sites required for their interaction with Atg8

and Atg11, and subsequent facilitation of their respective selective autophagy pathways. However, our studies have yet to determine which protein functions as the kinase that directly phosphorylates these selective autophagy receptors.

A recent study implicated Casein Kinase II (CK2) as the kinase that directly phosphorylates ScAtg32 at Serine-114 and Serine-119 to facilitate its interaction with Atg11. This implies that Slt2 most likely cannot also serve as the direct kinase of Atg32 at these residues. However, it is possible that Casein Kinase II functions downstream of Slt2 in phosphorylation of Atg32. In order to investigate this, CK2 could be co-expressed in the Δ *slt2* background to determine if the receptors interactions of Atg32 are restored. In addition, our studies have yet to determine whether Slt2 is the kinase that directly phosphorylates Atg36, at either the Atg8 binding site or Atg11 binding site.

After determining that Slt2 plays a role in phosphorylation of Atg32 and Atg36 in *S. cerevisiae*, we sought to identify a protein kinase that demonstrated similar effects on *P. pastoris* receptors. However, an Slt2 homolog has yet to be identified. A BLAST analysis revealed two top hits in the *P. pastoris* genome, one protein referred to as an “Slt2-like protein” in our results. A reverse BLAST analysis performed on the second *P. pastoris* protein revealed that it is most closely homologous to Smk1, another MAPK in *S. cerevisiae*. As the yeast two-hybrid system is in a *S. cerevisiae* background, it is a challenge to study how protein-protein interactions of heterologous yeast are affected by these kinases. As mentioned, Atg8 interaction only occurred in Atg30 when Serine-71 was mutated to Glutamate (Atg30^{S71E}). We postulated that nuclear co-expression of a kinase that is essential for phosphorylation of the receptor may facilitate Atg8 interaction. Interestingly, co-expression of both the Slt2-like protein and Smk1-like protein did not

result in interaction between Atg30 and Atg8 (Figure 13). The absence of interaction might be due to the following: (1) none of these kinases are the true Slt2 in *P. pastoris*, or (2) Slt2 is not the direct kinase of the receptors and the rest of *P. pastoris* downstream effector(s) are not present in the yeast two-hybrid assay. This likely prevents protein activation and regulatory events that would result in proper kinase activation to act on the *P. pastoris* receptor.

Surprisingly, nuclear co-expression of PpHrr25 did result in Atg30-Atg8 interaction. Hrr25 is a Casein Kinase I homolog that is also present in *S. cerevisiae*. This is a promising result that is consistent with the finding that PpAtg30 contains an AIM and Atg11 binding site that follows sequence homology with the consensus for casein kinases (Flotow *et al.* 1990; Kennelly and Krebs, 1991). Further investigation into the potential role of Hrr25 in phosphorylation of receptors in both *P. pastoris* and *S. cerevisiae* is warranted. Deletion of the HRR25 gene in both *S. cerevisiae* and *P. pastoris* produces a non-viable mutant, which presents a challenge for using it in yeast two-hybrid and in vivo studies. Therefore, a special technique must be implemented in order to study how Hrr25 affects selective autophagy pathways in yeast. A strategy for producing a viable *S. cerevisiae* $\Delta hrr25$ strain was described by Ravid and Hochstrasser, 2008. They deleted the wild-type gene in a strain that expresses an inducible and highly unstable form of the protein (Hrr25^{degron}). A similar strategy can be developed in *P. pastoris*, using the PpHrr25^{degron} and the methanol-inducible promoter. The Hrr25^{degron} can then be degraded over time to produce a true $\Delta hrr25$ mutant that can be subject to a pexophagy assay.

Additional yeast two- and three-hybrid experiments could be performed to explore the function of Hrr25 in *S. cerevisiae*. Much like with CK2, ScHrr25 could be

co-expressed in the Δ *slt2* background to determine if it restores receptor interactions lost in this mutant strain. This result would help determine if Hrr25 is downstream of Slt2.

The sites of Atg8 and Atg11 interaction for PpAtg30, ScAtg32, and ScAtg36 have now been determined. In this work, we demonstrate a common model for phosphoregulation of selective autophagy receptors and the mechanism by which they interact with Atg8 and Atg11 of the common autophagy machinery. However, further studies are necessary to understand how selective autophagy pathways are regulated, and which signaling pathways are involved. Additional experiments will be necessary to determine if Slt2, Hrr25, or another protein kinase is responsible for direct phosphorylation of PpAtg30 and ScAtg36. An in vitro kinase assay using purified kinases and substrates, obtained either via bacterial or yeast expression, would be an effective approach. In addition, further investigation into how these kinases are activated would lead to further understanding of the signaling and regulatory events that pexophagy, mitophagy, and other autophagy-related pathways in yeast and higher eukaryotes.

Acknowledgments

The Discussion section, in part, contains data and findings that have been published in a 2013 EMBO Reports article titled “Phosphorylation of pexophagy and mitophagy receptors coordinates their interaction with Atg8 and Atg11” [*EMBO Rep.* 14(5): 441-9]. The corresponding author Dr. Suresh Subramani and co-authors Dr. Jean-Claude Farré and Sarah Burnett have approved the inclusion of these findings in this work.

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IV. Materials and Methods

Yeast strains, plasmids, and transformations

All yeast two- and three-hybrid experiments were performed with the strains indicated in Table 2-2. A standard lithium acetate protocol as described was used to transform all *S. cerevisiae* yeast strains as previously described (Motley *et al.*, 2012). Cloning was performed using standard molecular biology methods. Site-directed mutagenesis of wild-type genes was performed using the QuikChange II Kit from Agilent Technologies.

Biochemical studies of pexophagy

For monitoring of pexophagy *in vivo*, the wild-type *S. cerevisiae* strain BY4742 and mutant strains with the BY4742 background (see Table 2-3) were grown (starting from 0.2 OD₆₀₀) in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) to an OD₆₀₀ of 1 at 30°C on a shaker set at 250 rpm. To induce peroxisome proliferation, they were shifted to oleate medium (1% oleate, 5% Tween-20, 0.25% yeast extract, 0.5% peptone, and 5mM phosphate buffer) starting from an OD₆₀₀ of 1. After 15 hours of growth in oleate, pexophagy was induced by shifting these cells to SD-N medium (0.17% Yeast Nitrogen Base without ammonium sulfate or amino acids and 2% glucose) at an OD₆₀₀ of 1. One milliliter of cells was collected at time points indicated in the figures, precipitated with trichloroacetic acid and analyzed by Western blot.

Yeast two- and three-hybrid assays

The *GAL4* based Matchmaker yeast two-hybrid system (Clontech Laboratories Inc.) was used. Full length open reading frames were inserted in pGAD-GH (AD) and pGBT9 (BD) plasmids, except for *P. pastoris* and *S. cerevisiae* Atg8, where the BD was fused to a truncated Atg8 (Methionine-1 to Phenylalanine-115). The pBridge plasmid from Clontech Laboratories (for co-expression of a BD fusion protein and a NLS fusion protein) was used exclusively for the three-hybrid assay. The *S. cerevisiae* strains AH109 and HF7c were used for the two- and three-hybrid assays, respectively. Two transformants from each strain were tested in duplicate in both assays. All strains were plated on SD medium (Leu⁻, Trp⁻) as well as SD medium (His⁻, Leu⁻, Trp⁻) containing 3-amino-1,2,4-triazole at the concentration indicated in the figure.

Table 2-1: Yeast strains used yeast two- and three-hybrid assays in this study

Strain	Description	Genotype	Reference
AH109	<i>S. cerevisiae</i> two-hybrid strain	<i>MATα</i> , <i>ura3-52</i> , <i>his3-200</i> , <i>ade2-101</i> , <i>trp1-901</i> , <i>leu2-3, 112</i> , <i>gal4Δ</i> , <i>met</i> , <i>gal80Δ</i> , <i>URA3::GAL1_{UAS}-GAL1_{TATA}-lacZ</i>	Clontech Inc.
$\Delta atg1$	AH109 background; <i>ATG1</i> deleted	AH109 + $\Delta atg1::G418$	This study
$\Delta slt2$	AH109 background; <i>SLT2</i> deleted	AH109 + $\Delta slt2::G418$	This study
HF7c	<i>S. cerevisiae</i> three-hybrid strain	<i>MATα</i> , <i>ura3-52</i> , <i>his3-200</i> , <i>lys2-801</i> , <i>ade2-101</i> , <i>trp1-901</i> , <i>leu2-3, 112</i> , <i>gal4-542</i> , <i>gal80-538</i> , <i>LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3</i> , <i>URA3::(GAL 17mers) 3-Cycl_{TATA}-lacZ</i>	Mitchell <i>et al.</i> , 2008 (Clontech Inc.)

Table 2-2: Yeast strains used for pexophagy assays

Strain	Description	Genotype	References
Sc WT	BY4742 + Thiolase-GFP	<i>MATα</i> ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>lys2Δ0</i> ; <i>ura3Δ0</i> <i>pot1::Thiolase-GFP (HIS5)</i>	Saleem <i>et al.</i> , 2008
Sc $\Delta atg36$	$\Delta atg36$ + Thiolase-GFP	BY4742 + $\Delta atg36::G418$	This study
Sc $\Delta atg1$	$\Delta atg1$ + Thiolase-GFP	BY4742 + $\Delta atg1::G418$	Saleem <i>et al.</i> , 2008

Table 2-3: Plasmids used in yeast two- and three-hybrid studies

Plasmid	Fusion Protein 1	Fusion Protein 2	Background	Marker	References
pGBT9	BD-empty	none	pGBT9	<i>TRP1</i>	Clontech
pGAD-GH	AD-empty	none	pGAD-GH	<i>LEU2</i>	Clontech
Pjcf589-F115	BD-PpAtg8	none	pGBT9	<i>TRP1</i>	This study
Pjcf226	AD-PpAtg30	none	pGAD-GH	<i>LEU2</i>	This study
Pjcf590	AD-PpAtg32	none	pGAD-GH	<i>LEU2</i>	This study
Pjcf659	BD-ScAtg8	none	pGBT9	<i>TRP1</i>	This study
Pjcf660	BD-ScAtg11	none	pGBT9	<i>TRP1</i>	This study
Pjcf652	AD-ScAtg32	none	pGAD-GH	<i>LEU2</i>	This study
Pjcf654	AD-ScAtg32 ^{S81AS83AS85A}	none	pGAD-GH	<i>LEU2</i>	This study
Pjcf656	AD-ScAtg32 ^{S114AS119A}	none	pGAD-GH	<i>LEU2</i>	This study
Pjcf653	AD-ScAtg36	none	pGAD-GH	<i>LEU2</i>	This study
Pjcf700	AD-ScAtg36 ^{S31A}	none	pGAD-GH	<i>LEU2</i>	This study
pJCF701	AD-ScAtg36 ^{S31E}	none	pGAD-GH	<i>LEU2</i>	This study
pJCF702	AD-ScAtg36 ^{S97A}	none	pGAD-GH	<i>LEU2</i>	This study
pJCF703	AD-ScAtg36 ^{S97E}	none	pGAD-GH	<i>LEU2</i>	This study
pBridge	BD-empty	none	pBridge	<i>TRP1</i>	Mitchell <i>et al.</i> 2008 (Clontech)
pJCF800	BD-ScAtg8	none	pBridge	<i>TRP1</i>	This study
pJCF801	BD-ScAtg8	NLS-ScAtg11	pBridge	<i>TRP1</i>	This study
pJCF804	BD-ScAtg11	NLS-ScAtg8	pBridge	<i>TRP1</i>	This study
pJCF805	BD-ScAtg8	NLS-ScSlt2	pBridge	<i>TRP1</i>	This study
pJCF806	BD-ScAtg11	NLS-ScSlt2	pBridge	<i>TRP1</i>	This study
pJCF807	BD-PpAtg8	NLS-PpHrr25	pBridge	<i>TRP1</i>	This study
pJCF808	BD-PpAtg8	NLS-PpSlt2	pBridge	<i>TRP1</i>	This study
pJCF809	BD-PpAtg8	NLS-PpSmk1	pBridge	<i>TRP1</i>	This study

Table 2-4: Plasmids used in pexophagy assays

Plasmid	Fusion Protein	Background Vector	Marker	References
pJCF500	HA-ScAtg36	pRS415	<i>LEU2</i>	This study
pJCF501	HA-ScAtg36 ^{F33AL36A}	pRS415	<i>LEU2</i>	This study
pJCF502	HA-ScAtg36 ^{S31A}	pRS415	<i>LEU2</i>	This study
pJCF503	HA-ScAtg36 ^{S97A}	pRS415	<i>LEU2</i>	This study

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