UNIVERSITY OF CALIFORNIA, SAN DIEGO

Atg36 Phosphorylation in *S. cerevisiae* and The Glucose Sensing Pathway

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by

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ABSTRACT OF THE THESIS

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Masters of Science in Biology

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General and selective autophagy are pathways eukaryotic cells can utilize to degrade damaged or superfluous cellular components, but they are also survival mechanisms during starvation. Therefore these degradation pathways can be activated by starvation conditions, typically carbon and/or nitrogen sources. We investigated the effects of nitrogen and carbon starvation on autophagy-related pathway; in particular we focused on peroxisome degradation (pexophagy) and the nutrient sensing pathways involved. Pexophagy in *Saccharomyces cerevisiae* requires the integration of two signals; one leading to activation, by phosphorylation, of the pexophagy receptor, Atg36, and a second to activate the core autophagy machinery. Atg36 phosphorylation depends on the presence of glucose, and the induction of core autophagy machinery depends on the inactivation of the Tor1 (Target of rapamycin) complex in the absence of nitrogen. The glucose signal has led us to the PKA (cAMP-dependent protein kinase A) signaling

pathway which seems to be directly or indirectly implicated in the phosphorylation of Atg36. This discovery has led to a deeper understanding of the effects of carbon and nitrogen on pexophagy in yeast that may apply to other selective autophagy pathways.

I:

Introduction

Autophagy

An organism's survival is dependent on its ability to function in and respond to changes in its environment. There are many internal mechanisms a cell can utilize to respond to changes in its extracellular environment, such as alterations in metabolic rate, temperature, and pH. In addition to controlling these internal parameters, organisms must be able to maintain homeostasis, both within their entire system and at a cellular level. Cellular homeostasis can be maintained in a myriad of ways; balancing biogenesis with the degradation or recycling of unnecessary or damaged organelles and other cytoplasmic components. One intracellular pathway eukaryotes utilize to degrade cellular contents is autophagy. This process, which literally means self-eating, was first described in 1966, when cellular components enveloped in vesicles were observed in mammalian cells (de Duve, Wattiaux, 1966). There are two major autophagy mechanisms; macroautophagy and microautophagy, both of which include the isolation and degradation of cytoplasmic materials or organelles (Figure 1) (Mizushima, 2007).

Both processes degrade cellular components in the lysosome (or vacuole in yeast) and release recycled building blocks, such as amino acids, back into the cytosol. The differences, however, are in how each pathway targets the cellular components for degradation in the lysosome or vacuole. In macroautophagy, large cargos are enveloped in a double membrane structure called an autophagosome, whereas in microautophagy, the lysosome or vacuole membrane itself invaginates and engulfs cytosolic components directly (Klionsky and Ohsumi, 1999). The degradation that occurs during autophagy plays two important roles within the cell. In addition to removing damaged and extraneous organelles, proteins or other cytoplasmic contents, autophagy also generates

macromolecular building blocks necessary to survive during periods of nutrient deprivation and cellular stress.

Selective autophagy

In addition to bulk degradation of damaged cytoplasmic components and organelles, the cell can also target only specific organelles or cargos for degradation in a mechanism called selective autophagy (Nazarko and Farre, 2014). While autophagy's nonselective method of intracellular degradation is a useful survival response in starvation conditions, selective autophagy enables the cell to remodel its intracellular makeup to best fit its extracellular environment. There are many types of selective autophagy, each of which targets a unique cellular component, such as mitochondria (mitophagy), the endoplasmic reticulum (ER-phagy), and peroxisomes (pexophagy). These selective autophagy mechanisms require the use of many general autophagy genes (the core autophagy machinery) in addition to their own specific adaptor proteins and receptors (Johansen and Lamark, 2011).

General mechanism and regulation of autophagy

Autophagy is a crucial cellular mechanism, necessary for cellular survival and viability. This process is so important, it has been found that many of the genes involved are conserved through many eukaryotic organisms (Klionsky *et al.*, 2003). Because of this conservation across organisms, *S. cerevisiae* has become a model organism to study this process and has helped determine the mechanisms of autophagy.

For autophagy to occur, the cell must first recognize that extracellular conditions necessitate the degradation of cellular components. In yeast, it is the Tor1 kinase complex that is responsible for extracellular nutrient availability and cellular stress

sensing (Cutler, 2001). Tor1 is sensitive and responds to extracellular levels of carbon, nitrogen, phosphate, or sulfate to induce autophagy. It is also a major protein regulator in cells and is responsible for many proteins. One of the proteins Tor1 effects is Atg13, a key component of the core autophagy machinery that is necessary for both general and selective autophagy. Atg13 is hyperphosphorylated by active Tor1 when there is an abundance of extracellular nutrients. Hyperphosphorylated Atg13 prevents the interaction of Atg13 and Atg1, a step necessary for autophagy to occur (Nair and Klionsky 2005). In the absence of one of the above stated nutrients, however, Tor1 is inactivated and there is a reduced phosphorylation of Atg13 and activation of Atg1 (Kamada, 2010).

Following the activation of Atg1, the phagophore membrane starts to elongate, from the phagophore assembly site (also known as the PAS), around targeted intracellular components (Abeliovich, 2000). Many Atg proteins are involved in this process, including many components of the PAS itself. These Atg proteins are collectively known as the core autophagy machinery and some of these proteins are the Atg1 kinase complex; Atg8 and Atg12, two ubiquitin-like proteins, and their associated conjugation systems; the Atg2-Atg18 complex; and Atg9, among others (Suzuki and Ohsumi, 2007). Once the selected cargo has been incorporated into a completed double-membrane vesicle known as the autophagosome, it is able to travel to, and fuse with, the vacuole to release its cargo (Wang, 2003). To release its cargo, the autophagosome's outer membrane fuses with the vacuole and the cargo, now surrounded by a single membrane (called the autophagic body), is introduced into the acidic vacuolar lumen. Inside the vacuole, hydrolases including the proteases, Pep4 and Prb1 (proteinase A and proteinase B, respectively), or lipases such as Atg15, break down the cargo into essential cellular

building blocks such as amino acids and lipid building blocks (Klionsky, 2005; Takeshige *et al.,* 1992; Farre and Subramani, 2004). These amino acids are finally exported back to the cytosol to be reused for more crucial purposes by amino acid exporters such as Atg22 (Yang *et al.*, 2006).

Selective autophagy specificity via receptors

For selective autophagy to occur, targeted organelles must be marked to enter the autophagic cycle in addition to the activation of the cell's core autophagy machinery. Across all eukaryotes, organelles are tagged for degradation by the activation of key selective autophagy receptors associated with the organelle membrane. These receptors in yeast, which are always present on the organelle surface, are activated to induce organelle degradation via phosphorylation (Farre, 2008; Stolz, 2014). In *S. cerevisiae* mitochondria, Atg32 is the specific receptor responsible for mitophagy. Phosphorylated Atg32 binds to Atg11 and Atg8 to induce the degradation of the mitochondria (Kanki *et al.,* 2009, Okamoto *et al.*,2009). Another form of selective autophagy is ER-phagy, a processes that maintains cellular homeostasis by monitoring and maintaining ER size within the eukaryotes (Schuck, 2014). Atg39 and Atg40 are ER proteins in yeast that are implicated in ER-phagy (Mochida, 2015). One more type of selective autophagy in cells is pexophagy, the specific degradation of peroxisomes within a cell. In *S. cerevisiae*, the Atg36 (Autophagy Related Gene 36) protein is the receptor that is phosphorylated to induce pexophagy (Till, 2011; Motley, 2012).

Tracking selective autophagy

Just as autophagy frees up unnecessary or damaged cellular components to be utilized by the cell, as autophagy progresses, its machinery can be recovered and reused

for the degradation of other cellular components. The core autophagy machinery's ability to be reused to recycle another cellular component after helping form the PAS and the autophagosome is a crucial aspect of autophagy (Inoue, 2010). One protein that is recovered for subsequent vesicle formation is Atg8. Atg8 associates with the membrane of the expanding phagophore as the vesicles are created, but are cleaved from the outer phagophore membrane via the action of the Atg4 protein, before the structure becomes the autophagosome. However, some Atg8 associated with the inner phagophore membrane is left in the autophagosome and is degraded in the lysosome or vacuole during autophagy. Hence, autophagy can be monitored by tracking Atg8 delivery to, or degradation in, the vacuole or lysosome.

To track selective autophagy, proteins unique to each organelle can be used in a similar manner. For example, OM45 is a mitochondrial surface protein, located on the mitochondrial outer membrane. Because of OM45's location, it is a prime protein to use to track mitophagy. By fusing OM45 with GFP, one can track the mitochondria's position. As the mitochondria are degraded in the vacuole during mitophagy, GFP is cleaved from OM45-GFP and remains stable in the lysosome. The levels of free GFP released from the OM45-GFP fusion can be used to track mitophagy levels (Kanki, 2009). The same method is used to track ER-phagy. Sec61 is an ER membrane protein that can be fused to GFP. Just as levels of free GFP cleaved from OM45-GFP can be used to measure levels of mitophagy, levels of ER-phagy can be tracked by monitoring levels of free GFP cleaved in the vacuole from Sec61-GFP. Pexophagy can also be tracked in a method similar to those used to track general autophagy, mitophagy and ER-phagy. Pot1(thiolase) is a peroxisomal matrix protein that GFP can be fused to. Just as we have

previously seen, Pot1-GFP is degraded to Pot1 and free GFP when pexophagy occurs. With the use of this method, one can observe selective or general autophagy as it occurs by monitoring levels of free GFP within a cell.

Pexophagy

Pexophagy is a specific form of selective autophagy that is utilized when excessive amounts of peroxisomes are detrimental rather than beneficial to cells. For example, pexophagy occurs when cells are moved from a medium which contains a carbon source that requires peroxisome metabolism (and a large number of peroxisomes) to a medium which does not require peroxisome function (and thus, a minimal amount of peroxisomes) (Till, 2011). For an organelle as crucial as the peroxisome, degradation must be tightly regulated to ensure the cell is responding to the environment in the best possible way it can. Pexophagy only occurs when there are superfluous or damaged peroxisomes in the cell and is therefore a tightly regulated process (Motley, 2012). If the cell decides degradation of peroxisomes is necessary, it is not until Atg36 is phosphorylated by the kinase, Hrr25, that pexophagy can begin (Tanaka, 2014). Once Atg36 is phosphorylated, the peroxisome is prepared for pexophagy by interacting with general and selective autophagy factors (Farre, 2013).

Nutrient sensing within the cell and the core autophagy machinery

A cell's ability to sense its extracellular environment and adapt to eliminate any extraneous organelles is essential to its survival. Of all the responses a cell has to starvation conditions, the activation its core autophagy machinery is the most crucial for any type of degradation to occur. Although general autophagy can be influenced by either nitrogen, carbon, phosphate, or sulfate starvation, different forms of selective autophagy

occur only in certain starvation conditions. For example, generally the conditions that require peroxisome metabolism, such as oleate medium, will stimulate proliferation of these organelles and a switch to a different carbon source, such as glucose or ethanol, that does not require peroxisome metabolism, in addition to nitrogen starvation, will induce their degradation by pexophagy.

The nitrogen sensing pathway plays a crucial role in pexophagy. It is responsible for the activation of the core autophagy machinery, and is necessary for peroxisome degradation as well. The major cellular regulator, Tor1 kinase, is the main component of this pathway. As mentioned previously, the presence of nitrogen keeps Tor1 active. Tor1's activity leads to the activation of many proteins, one of which is the kinase, Sch9. Sch9 itself regulates many proteins as well, including the cell's autophagy machinery. The cell's core autophagy machinery is kept inactive by Sch9, which is activated by Tor1. Therefore, in the presence of nitrogen, the autophagy machinery is repressed. However, when nitrogen is absent, Tor1 and Sch9 are no longer active, thus the cell's general autophagy machinery is free to degrade cellular components. The nitrogen sensing pathway can be tracked by monitoring the phosphorylation of S6. S6 is a ribosomal protein downstream of Tor1 and Sch9 and when both are active (along with the rest of the nitrogen sensing pathway), S6 is phosphorylated.

Pexophagy is also regulated by glucose. The glucose sensing pathway is a complex one, full of many different regulators. Of all glucose regulators within *S. cerevesiae*, Dr. Jingjing Liu has narrowed it down to the Gpr1-Gpa2 pathway; she found a *gpr1* and *gpa2* double deletion has a significant delay in pexophagy (unpublished data). The Gpr1-Gpa2 pathway and Ras2 sense nutritional signals and regulate adenylyl cyclase

(Cdc35) activity and consequently cAMP synthesis, leading to the activation of PKA (Protein Kinase A). Interestingly, one of PKA's roles as a glucose regulator is to also inactivate the core autophagy machinery when glucose is available. Upon PKA's inactivation, the autophagy machinery is no longer repressed and can be utilized for selective and general autophagy, including pexophagy, similar to the effects of Sch9 inactivation (Zaman, 2009). Additionally, PKA can be regulated by Tor1, as well as glucose.

In the absence of nitrogen, Tor1 does not activate PKA and Sch9, both of which, when active, would inhibit autophagy, thus, autophagy can be induced (Budovskaya, 2004). But the similarities between PKA and Sch9 extend beyond the same autophagy proteins they both inhibit and Tor's effect on them. In the absence of either Sch9 or PKA, the remaining kinase can replace the other's roles in the cell (Toda, 1988). This extensive overlap between Sch9 and PKA in the glucose and nitrogen sensing pathways and autophagy machinery inhibition make these pathways very interconnected and fine tuned (Figure 2) (Yorimitsu, 2007).

Although we know much about pexophagy, there are still gaps in our knowledge left to fill. The interactions of phosphorylated Atg36 with the general and specific autophagy machinery are understood, but the detailed pathway triggering Atg36 phosphorylation remains to be sorted out. Interestingly, glucose and nitrogen inhibit the core autophagy machenery, but glucose is also necessary to induce pexophagy. Understanding how the presence of glucose and starvation for nitrogen are both necessary for pexophagy is the last piece to the puzzle which would allow us to better understand pexophagy both as an individual process and a component of a cell's overall functioning

and is what I studied. Although pexophagy is a very specific process, understanding it is a crucial step to fully grasp how the cell copes with changes in its extracellular environment. The importance of such a global aspect of cellular functioning cannot be overstated, and because this process is conserved across all eukaryotes, understanding it would provide universal benefits to the biological field.

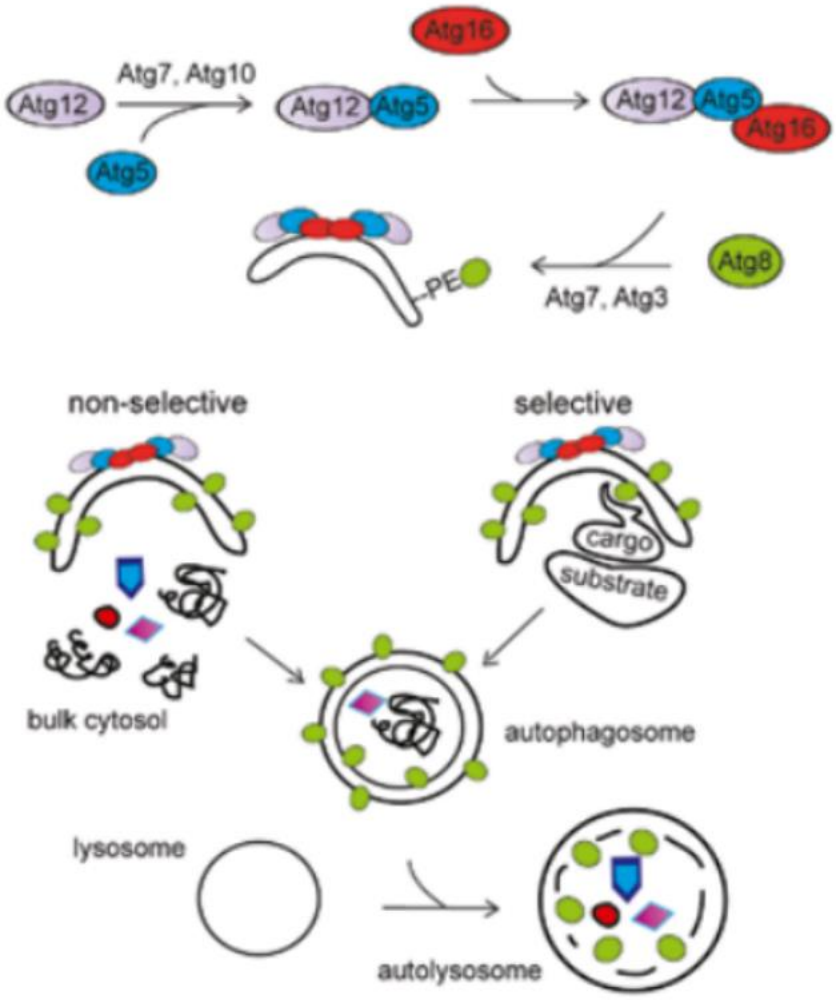


Figure 1. Schematic depiction of macroautophagy and microautophagy; the two main types of autophagy in yeast (Popovic, 2012). In macroautophagy, the autophagosome is formed as the expanding phagophore sequesters cytoplasmic components and select organelles to create the autophagic body. This autophagic body is introduced to the vacuole lumen as the autophagosome fuses with the vacuole membrane where it can be degraded by vacuolar hydrolases. In microautophagy, however, cellular cargo are taken up by the vacuole by invagination of its membrane to be degraded.

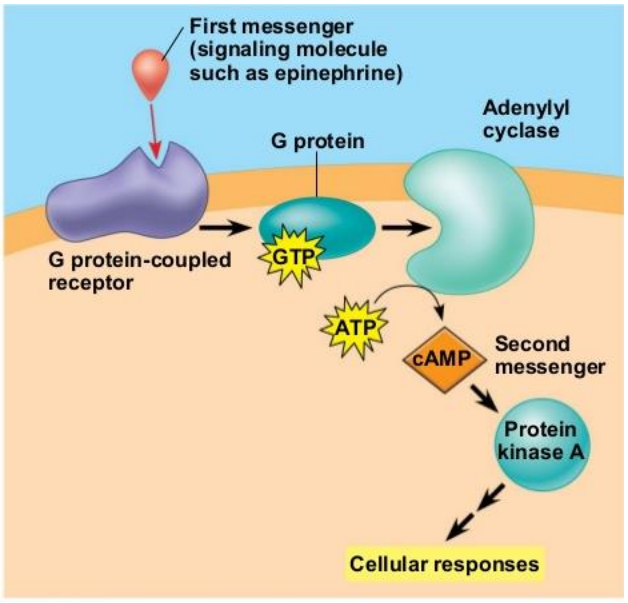


Figure 2. Glucose signaling in yeast (Campbell, 2011, ch. 11). Glucose signaling in yeast cells is a complex pathway that has many different branches, one of which includes PKA (Protein Kinase A) to induce the cellular responses necessary. In autophagy, these cellular responses include the activation of the core autophagy machinery.

II:

Results

General Autophagy can occur in either carbon or nitrogen starvation conditions

I began this project by confirming which starvation conditions trigger general autophagy. General autophagy uses machinery also necessary for selective autophagy, so by understanding the conditions in which general autophagy can occur, we can better understand selective autophagy. Most of the autophagy related processes require the protein Atg8, a component of the autophagosome. During general autophagy, the autophagosome (and the proteins and cellular components associated with it) are transported to the vacuole to be degraded. This process can be tracked with GFP-Atg8. Once the autophagosome is in the vacuole, the vesicle is digested, a process which includes the cleavage of GFP from GFP-Atg8. As fully folded GFP is relatively resistant to vacuolar proteases, general autophagy can be monitored by measuring the accumulation of free GFP in a cell during autophagy induction (Figure 3).

General autophagy was observed in nitrogen (N) and carbon (dextrose/glucose, D) starvation conditions. The nitrogen starvation condition (+D-N) is synthetic defined medium (SD) without either ammonium sulfate or amino acids and the carbon starvation condition (-D+N) is SD medium without dextrose. Over the span of six hours, there was an increase in free GFP in both nitrogen and carbon starvation conditions. However, there was more free GFP over the course of the experiment in nitrogen starvation conditions compared to carbon starvation, indicating that nitrogen starvation induces autophagy more strongly or rapidly than carbon starvation alone. In addition to measuring GFP cleavage from GFP-Atg8, we measured Phospho-S6 (PS6), an indicator of active Tor1. PS6 was present only at time point zero, indicating Tor1 was active in the medium the cells were moved from, SD. However, by two hours, there was no more PS6 because

Tor1 was rapidly inactivated in both samples once they were moved into starvation conditions. In addition to measuring PS6 and free GFP levels, actin was measured as a loading control.

Organelle-selective autophagy requires glucose

Next, we set out to determine if selective autophagy occurs in the same conditions as general autophagy. Selective autophagy requires general autophagy (core) machinery in addition to machinery specific to each individual form of specific autophagy. These components, specific to each form of selective autophagy, may be induced in conditions different from those in which general autophagy can occur, so it is crucial to understand what nutrients, or lack of them, play a role in these cellular processes.

A. Mitochondria are not degraded during carbon starvation

The first form of selective autophagy we investigated was mitophagy. Mitophagy, the targeted breakdown of the mitochondria, uses several proteins in addition to those necessary for general autophagy, one of which is the receptor Atg32. We used the cleavage of GFP from OM45-GFP, a mitochondrial membrane protein of unknown function, to measure mitophagy (Figure 4). Cells were moved from yeast extract peptone lactose (YPL) medium to either nitrogen or carbon starvation conditions, but unlike GFP-Atg8, there was cleavage of GFP from OM45-GFP primarily in nitrogen starvation conditions. When samples were analyzed directly from YPL medium, S6 was not phosphorylated (data not shown). However, PS6 was present at time point zero, but was rapidly lost over time in either medium. The presence of PS6 at time zero (which often is

for a few minutes in the new medium) indicates that Tor1 was activated at time zero, and S6 was phosphorylated, when either glucose (+D-N) or nitrogen (-D+N) was added.

B. ER degradation is not induced by carbon starvation

To measure ER degradation, we used Sec61-GFP, an ER resident protein. An increase in cleavage of GFP from Sec61-GFP over time indicated degradation of the ER in the vacuole during ER-phagy (Figure 5). After being moved to either nitrogen or carbon starvation conditions from SD, GFP cleavage was observed only in nitrogen starvation conditions, as indicated by the steadily increasing levels of free GFP throughout the duration of the experiment. PS6 levels were also only present at the zero time point and had dissipated by the second hour and remained unphosphorylated for the remainder of the experiment. Tor1's initial activity was from the SD medium cells were moved from but was inactivated in both starvation conditions, eventually freeing the general autophagy machinery it usually represses. Once again, actin was used as a loading control.

C. Pexophagy occurs only in nitrogen starvation conditions

To measure pexophagy, we used Pot1-GFP, a peroxisomal matrix protein. During pexophagy, GFP is cleaved from Pot1-GFP in the vacuole and levels of free GFP increase (Figure 6). In this experiment, cells were moved from oleate medium to either carbon or nitrogen starvation conditions. Oleate medium was used to induce peroxisome proliferation. Similar to both mitophagy and ER-phagy, there was GFP cleavage only in the presence of glucose and the absence of nitrogen- there was no pexophagy in the absence of glucose. Similar to the YPL condition, we know S6 is not phosphorylated in oleate medium and Tor1 is inactive. However, in the presence of dextrose, Tor1 is

quickly activated, as reflected by the faint PS6 band at time point zero. However, the nitrogen starvation conditions are not enough to keep Tor1 activated and Tor1 is quickly inactivated again, as can be seen in the lack of PS6 at the other time points.

These results indicate that dextrose plays a unique role in selective autophagy. In addition to the necessary core machinery active only in nitrogen or carbon starvation conditions, the presence of dextrose affects the cells in a way that triggers selective autophagy. Whether glucose marks certain organelles for degradation or its presence manipulates conditions within the cell that activate machinery necessary for selective autophagy different from general core autophagy machinery, the details are yet to be investigated in all the different forms of selective autophagy. In pexophagy however, the presence of glucose may be implicated in the phosphorylation of the pexophagy receptor Atg36, when phosphorylated, tags the peroxisome for degradation.

Atg36 phosphorylation with or without glucose

Atg36 is a *S. cerevisiae* protein which localizes at the peroxisome membrane and must be phosphorylated for pexophagy to occur. The phosphorylated Atg36 performs its pexophagy receptor function by recruiting components of the autophagic machinery, Atg8 and Atg11. Atg36 is phosphorylated in the presence of dextrose (glucose) and is not phosphorylated in oleate medium. Jingjing Liu, a former member of the Subramani Lab, investigated if Atg36 can be phosphorylated in oleate medium if dextrose was present (Figure 7). Atg36 phosphorylation was followed with an HA-Atg36 fusion protein; an upward shift in the protein over time indicated the phosphorylation of Atg36. In the presence of dextrose, there was a disappearance of the lower, unphosphorylated HA-Atg36 band paired with the appearance of the higher, phosphorylated HA-Atg36 band

over the hour. In contrast, in oleate medium alone, most of the unphosphorylated HA-Atg36 band remained present throughout the experiment, despite the phosphorylation of a small portion of the proteins. However, the HA-Atg36 phosphorylation levels in the presence of dextrose were significantly greater than the phosphorylation levels in oleate medium. Just as we observed when tracking pexophagy, oleate medium is a poor carbon source that inactivates Tor; therefore, there was no PS6 in the oleate medium. Oleate medium with 2% dextrose, however, did have S6 phosphorylation.

Atg36 phosphorylation is dependent on the carbon source

HA-Atg36 is known to be phosphorylated in the presence of dextrose but not in the presence of oleate, both of which are carbon sources. To understand this discrepancy, Jingjing Liu went on to investigate other carbon sources in which Atg36 could be phosphorylated (Figure 8). Cells were moved from oleate medium to nitrogen starvation conditions with a carbon source of either dextrose (+D-N), 2deoxyglucose (2DG-N), glycerol (Gly-N), ethanol (Eth-N), or galactose (Gal-N), or both nitrogen and carbon starvation (-D-N). Interestingly, in normal pexophagy condition (+D-N) Atg36 modification started by shifting to a lower molecular size (at 15 minutes) and then to the fully phosphorylated form after 60 minutes. To measure HA-Atg36 phosphorylation, the appearance of the lower band, then the replacement of the lower bands by the higher molecular weight phosphorylated HA-Atg36 band were considered. Out of all six conditions, glucose triggered the greatest level of HA-Atg36 phosphorylation and pexophagy. In addition of glucose, only 2-deoxyglucose and ethanol induced the appearance of HA-Atg36 of lower molecular weight. However, only ethanol showed a slight increase in HA-Atg36 phosphorylation paired with the greatest disappearance of

unphosphorylated HA-Atg36 (after dextrose). Only dextrose, and to a lesser extent, ethanol conditions had a slight increase in PS6 levels that disappeared by two hours. The phosphorylation of both HA-Atg36 and S6 in the dextrose and ethanol samples was coupled with the cleavage of GFP from Pot1-GFP over the course of the experiment. The cleavage of GFP in the dextrose sample was much greater than that of the ethanol sample, echoing the HA-Atg36 phosphorylation patterns of the samples. There was no pexophagy in any other condition.

Roles of the glucose and nitrogen sensing pathways on Atg36 phosphorylation

PKA inactivation does not reduce Atg36 phosphorylation

We wanted to see if disturbing the glucose sensing pathway would alter Atg36 phosphorylation patterns (Figure 9). PKA is a component of the glucose sensing pathway in *S. cerevisiae.* It is essential for growth so the *PKA* gene cannot be deleted. Instead, we used a mutant strain that disrupts PKA's activity in the presence of a drug (1NM-PP1). This is called an analog sensitive strain. We grew cells in SD medium overnight and moved them to nitrogen and carbon starvation conditions (-D-N) while inactivating PKA with the drug 1NM-PP1 (at a concentration of 100uM). After an hour with the drug, dextrose was added (+D-N). Over the span of an hour, the drug inactivated PKA strain had Atg36 phosphorylation patterns similar to that of the wild type. However, unlike the wild type, S6 phosphorylation was completely blocked in the PKA inactivated strain, reflective of the fact that when *S. cerevisiae* are moved from a poor carbon source to a good one, it is mostly PKA that is responsible for S6 phosphorylation.

Inactivation of Sch9 does not inhibit Atg36 phosphorylation

We also manipulated the nitrogen sensing pathway to see if that would alter Atg36 phosphorylation patterns. Sch9 is a component of the nitrogen sensing pathway in *S. cerevisiae*. Sch9 is also essential for growth so we used an analog sensitive strain to inactivate it, identical to the protocol used to inhibit PKA. It is known that nitrogen is not implicated in Atg36 phosphorylation; Atg36 can be phosphorylated in nitrogen starvation conditions. Therefore, one would not expect a component of the nitrogen sensing pathway to have a role in Atg36 phosphorylation. However, in the cell, Sch9 can compensate for the absence of PKA and vice versa, so one must examine each protein's individual role in Atg36 phosphorylation. Over one hour, the drug inhibited Sch9 strain had HA-Atg36 phosphorylation patterns similar to that of the wild type. Similarly, S6 phosphorylation patterns were comparable to that of the wild type, probably because PKA is responsible for phosphorylating S6 under these conditions.

Inactivation of both PKA and Sch9 delays Atg36 phosphorylation

Individually, the glucose sensing pathway or the nitrogen sensing pathway had no effects on Atg36 phosphorylation, but when inactivated together, they slightly changed the Atg36 phosphorylation pattern (Figure 11). Similar to the previous two experiments, the cells were grown overnight in SD medium and moved to carbon and nitrogen starvation conditions for an hour with the drug 1NM-PP1 to inhibit both PKA and Sch9. Dextrose was then added (+D-N) to initiate the experiment. With both PKA and Sch9 inactivated, we finally saw effects on HA-Atg36 phosphorylation; there was a delay over the span of an hour. Similar to the wild type, there was a dephosphorylation of Atg36 by 8 minutes, but then the protein remained dephosphorylated for almost the remainder of

the experiment, unlike the wild type, which was rapidly phosphorylated following its dephosphorylation. Additionally, we saw less S6 phosphorylation than in the wild type.

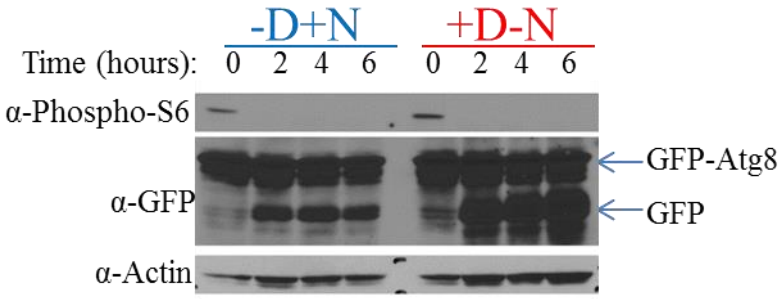
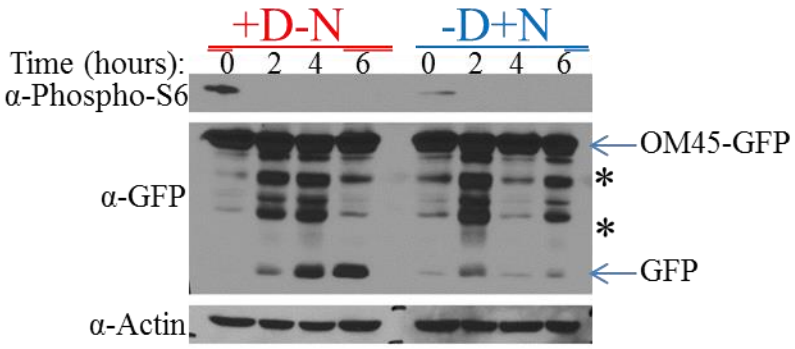


Figure 3. Carbon or nitrogen starvation conditions induce general autophagy. Wild type cells expressing GFP-Atg8 grown overnight in SD medium were move to the minimal medium lacking either dextrose (D) or nitrogen (N) for six hours to induce general autophagy. PS6 levels were used to measure Tor1 activity, GFP cleavage from GFP-Atg8 was used to monitor general autophagy and actin was used as a loading control.



*non-discriminate binding

Figure 4. M**itophagy occurs only during nitrogen starvation conditions.** Wild type cells expressing OM45-GFP grown overnight in SD-His medium were moved to either carbon or nitrogen starvation conditions for six hours to determine if mitophagy can occur in the same conditions as general autophagy. PS6 levels were monitored to measure Tor1 activity, GFP cleavage from OM45-GFP was monitored with α GFP antibodies, and actin was used as a loading control.

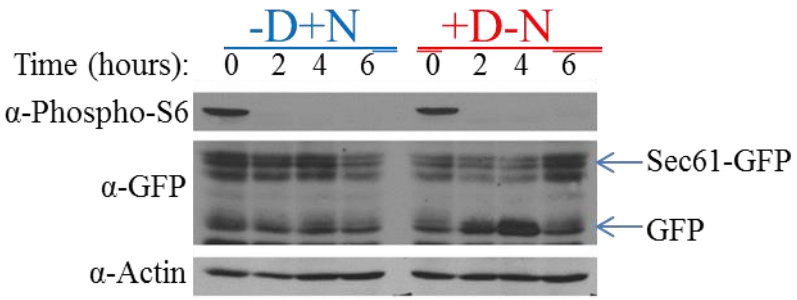


Figure 5. ER-phagy occurs only during nitrogen starvation conditions. Wild type cells expressing Sec61-GFP grown overnight in SD-His medium were moved to either dextrose or nitrogen starvation conditions for six hours to determine if ER-phagy can occur in the same conditions as general autophagy. PS6 levels were monitored to measure Tor1 activity, GFP cleavage from Sec61-GFP was monitored with α GFP antibodies, and actin was used as a loading control.

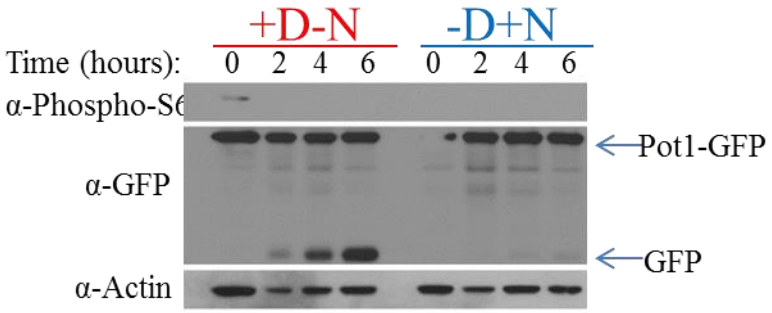


Figure 6. Pexophagy can only occur in the presence of glucose and the absence of nitrogen. Wild type Pot1-GFP cells grown overnight in oleate medium were moved to minimal medium, lacking either dextrose or nitrogen for six hours to observe if pexophagy can occur. S6 phosphorylation levels were used to measure Tor1 activity, GFP cleavage from Pot1-GFP was used to measure pexophagy and actin was used as a loading control.

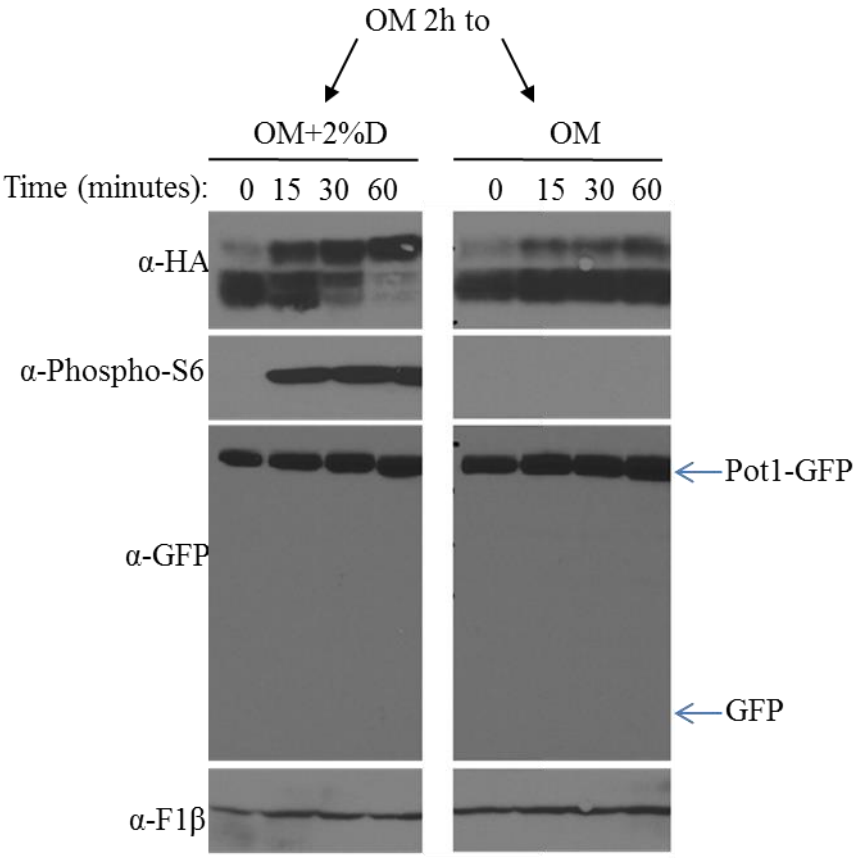
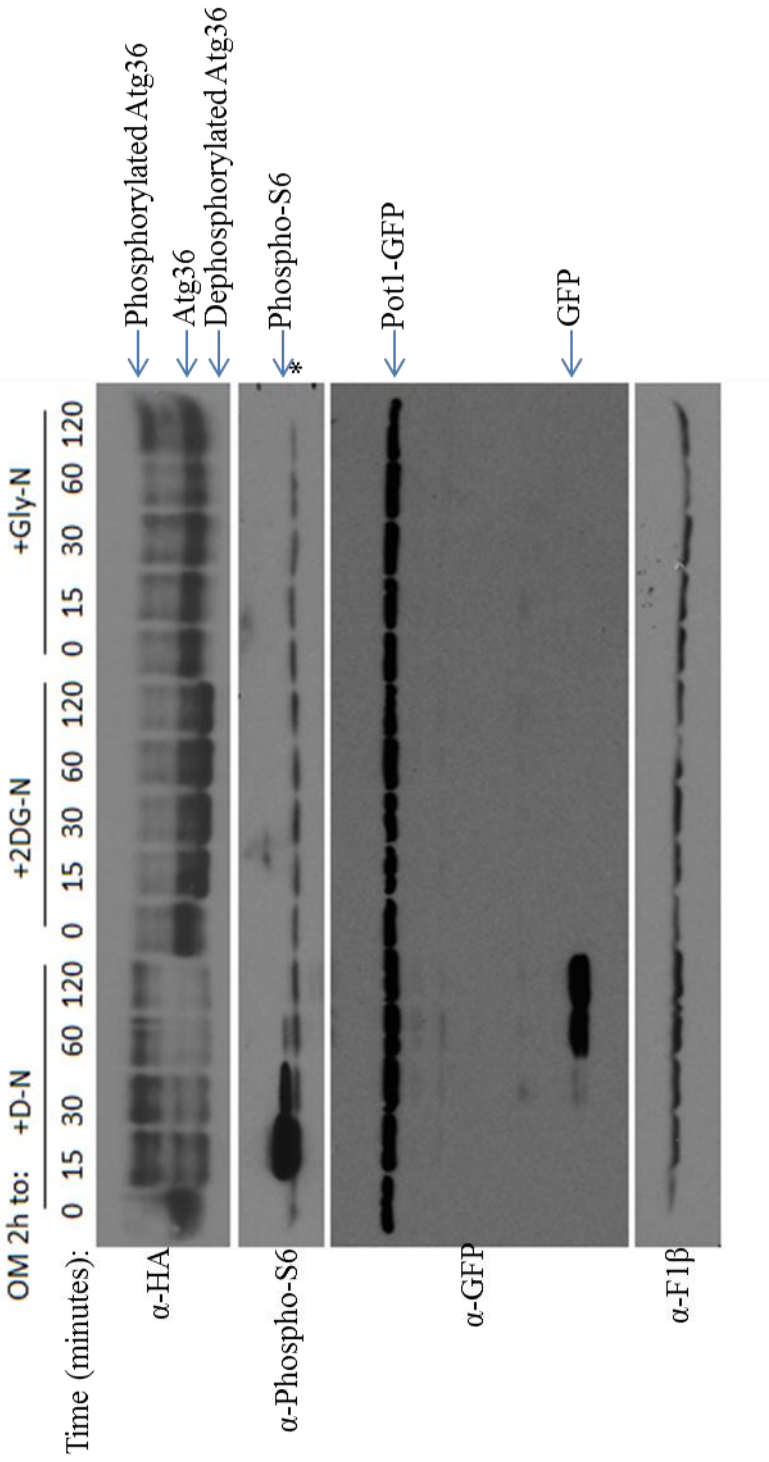


Figure 7. Atg36 is phosphorylated in oleate medium if dextrose is present. Wild-type cells grown in oleate medium for two hours were split into two cultures and 2% glucose was added in one of them. Phosphorylation of Atg36 was rapidly induced by glucose. HA antibodies were used to track the phosphorylation of HA-Atg36, antibodies against PS6 were used to measure Tor1 activity, Pot1-GFP was used to track pexophagy through GFP cleavage, and F1β was used as a loading control.

Figure 8. Glucose is the best carbon source for the induction of pexophagy. Cells grown in oleate for two hours were moved to nitrogen starvation conditions with five different carbon sources for two hours to determine if they have an effect on Atg36 phosphorylation. Anti-HA antibodies were used to determine the phosphorylation levels of HA-Atg36. S6 phosphorylation was measured with anti PS6 antibodies to determine Tor1 activity, GFP antibodies were used to measure levels of pexophagy and F1β was used as a loading control.



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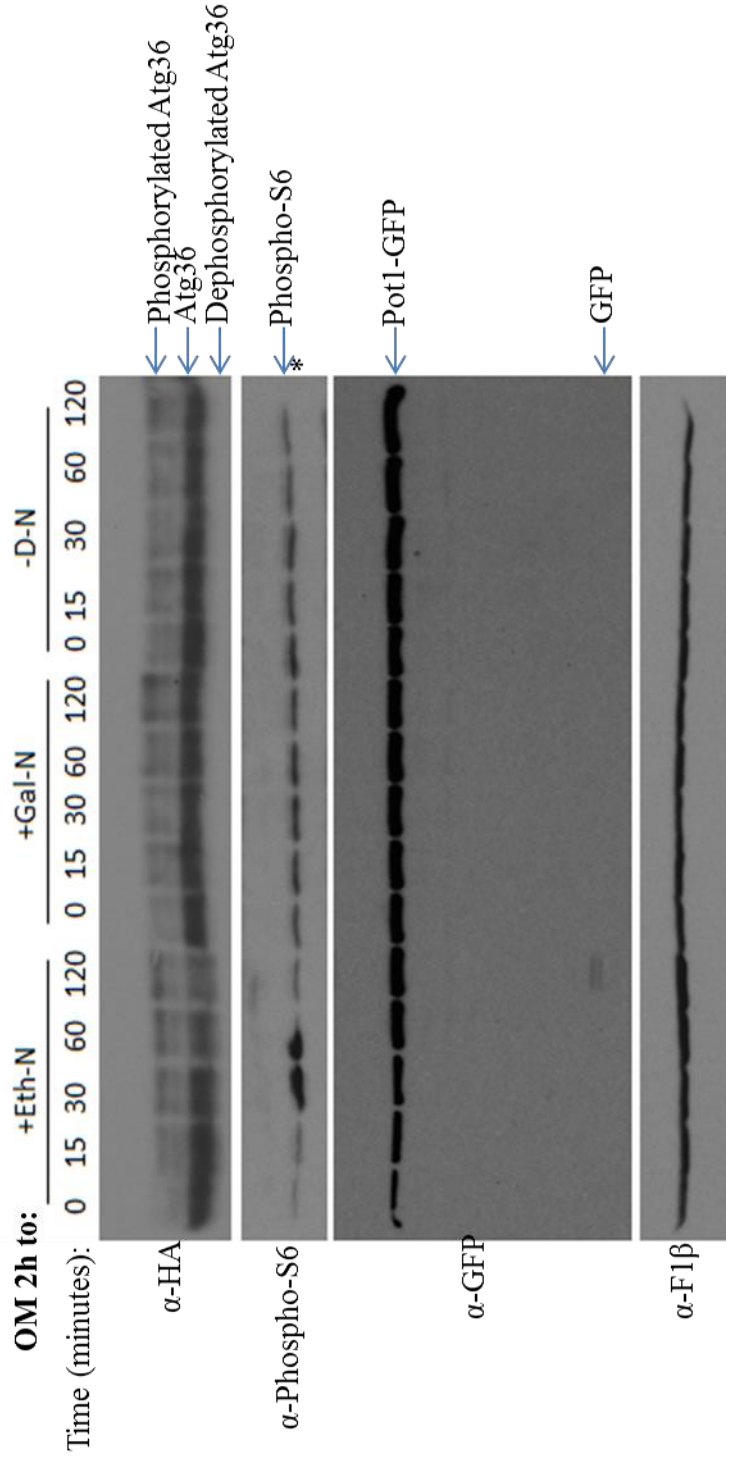

Figure 8., continued

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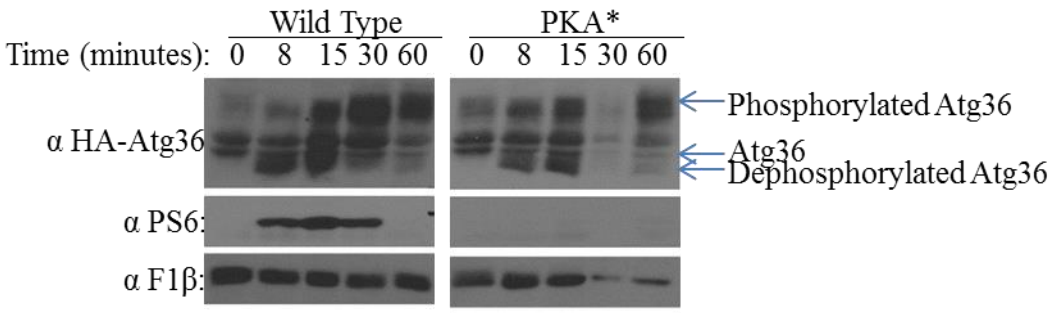

<u>Figure 9.</u> The inactivation of PKA alone does not effect levels of Atg36 phosphorylation. Cells grown in oleate for two hours were moved to SD-N medium for an hour to determine if Atg36 phosphorylation could be induced after PKA had been inactivated. 1NM-PP1 was used at a concentration of 100 uM to inactivate PKA. PS6 antibodies were used to measure Tor1 activity, anti HA antibodies were used to follow HA-Atg36 phosphorylation, and F1β was used as a loading control.

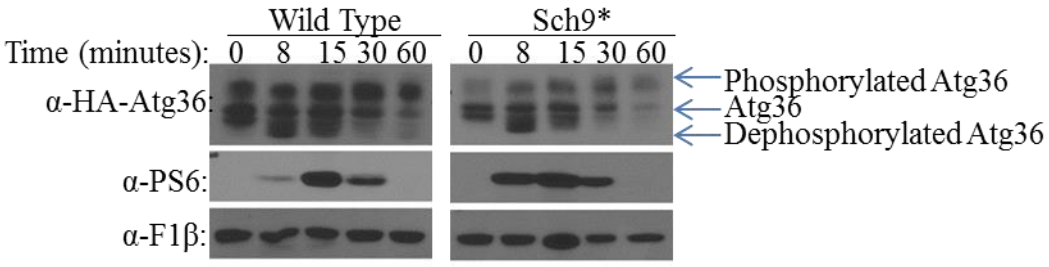

Figure 10. The inactivation of Sch9 alone is not enough to prevent Atg36 phosphorylation. Cells grown in oleate for two were moved to SD-N medium for an hour to determine if Atg36 phosphorylation could be induced after Sch9 had been inactivated. 1NM-PP1 was used at a concentration of 100 uM to inactivate Sch9. PS6 antibodies were used to measure if Sch9 was active or not, anti HA antibodies were used to follow HA-Atg36 phosphorylation, and F1β was used as a loading control.

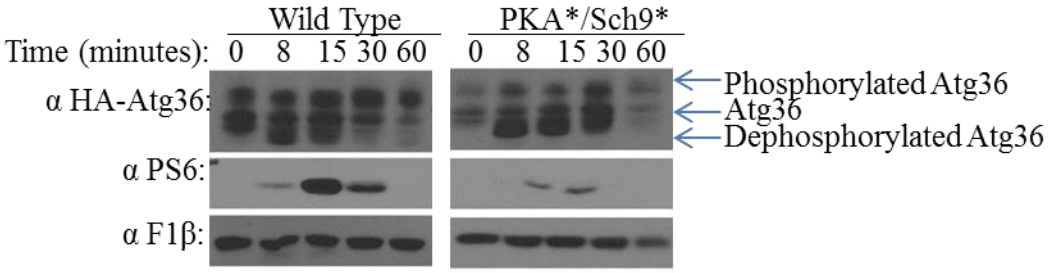

Figure 11. **The inactivation of PKA and Sch9 together delays Atg36 phosphorylation.** Cells grown in oleate for two hours moved to SD-N medium for an hour to determine if Atg36 phosphorylation could be induced after both Sch9 and PKA had been inactivated. 1NM-PP1 was used at a concentration of 100 uM to inactivate PKA and Sch9. PS6 antibodies were used to measure if Sch9 was active or not, anti HA antibodies were used to follow HA-Atg36 phosphorylation, and F1β was used as a loading control.

III:

Discussion

In this work, we have investigated the roles of the nitrogen and carbon sensing pathways in pexophagy. An examination of the effects of these nutrients, or lack of them, on cellular core autophagy machinery, along with analysis of the effects of different forms of carbon on the phosphorylation of the pexophagy receptor of *S. cerevisiae*, Atg36, has contributed to the greater understanding of the signaling events that initiate pexophagy.

Activation and utilization of core autophagy machinery in starvation conditions

When investigating the effects of nitrogen or carbon starvation on various forms of autophagy, we sought to identify which starvation conditions were necessary for mitophagy, ER-phagy, and pexophagy as well as general autophagy. It is known that general autophagy occurs in the absence of either nitrogen and/or carbon, while pexophagy occurs only in nitrogen starvation conditions, but we wanted to know more about other forms of selective autophagy. Because general autophagy requires just the activation of cellular core autophagy machinery for degradation, while selective autophagy also requires the selection of specific organelles for degradation, we predicted that mitophagy and ER-phagy would need one of the two nutrients for degradation to occur. We observed that mitophagy, ER-phagy, and pexophagy all occur only in both the presence of glucose and the absence of nitrogen.

Analysis of general autophagy indicated that general autophagy occurs in both nitrogen and carbon starvation conditions, although more degradation occurs in nitrogen starvation conditions (Figure 3). This degradation is dependent upon the activation of the core autophagy machinery. As the myriad of autophagy related proteins are activated, they are able to congregate around cytosolic components to form the PAS and initiate the

creation of the phagophore, with which autophagy can occur. These core autophagy proteins, which include Atg 1, Atg13, and Atg29, among others, are all under the control of Tor1. In the absence of either nitrogen or carbon, there is inactivation of Tor1, and thus, the activation of the proteins necessary for autophagy (Figure 3).

Selective autophagy, however, requires more than just the activation of the cell's autophagy machinery. To degrade specific cellular components, targets must be marked for degradation, in addition to the activation of autophagy machinery. Therefore, selective autophagy can occur in conditions more specific than general autophagy, but can never occur in conditions that autophagy cannot.

In mitophagy, ER-phagy, and pexophagy, there is GFP cleavage only in the absence of nitrogen, but not in glucose starvation, conditions (Figure 4, 5, and 6, respectively). In every one of these cases, the autophagy machinery is active. In all experiments, PS6 was present only at time point zero, if it was present at all, indicating that Tor1 was active in the beginning of the experiment, but was rapidly inactivated in both starvation conditions, activating general autophagy machinery. However, the fact that these selective autophagy pathways do not occur in the absence of glucose, which also activates the general autophagy machinery (Figure 3), suggests that glucose plays an important role in selective autophagy.

Carbon and Atg36 phosphorylation

After confirming general autophagy machinery is active and possible in both nitrogen and carbon starvation conditions, but pexophagy occurs only upon nitrogen starvation but requires glucose, we investigated the glucose signaling pathway for peroxisomes to be marked for degradation. In *S. cerevisiae*, the receptor responsible for

pexophagy is the peroxisomal membrane protein Atg36. Atg36 must be phosphorylated for pexophagy to occur, so to understand if it is specifically the presence of carbon or absence of nitrogen that plays a role in pexophagy, we had to delve deeper into its phosphorylation process.

Jingjing Liu, a former post-doc from the Subramani lab found that in the presence of glucose, Atg36 is phosphorylated in a two step process, although the mechanism through which this occurs is still unknown (Jingjing Liu, unpublished). First, Atg36, which has some basal levels of phosphorylation, is stripped of its phosphate groups to a dephosphorylated state, which is then able to be completely phosphorylated to its active state. It is also known, that the glucose sensing pathway reacts rapidly to the presence of glucose in extracellular environments (Tuttle, 1995). It has also been observed that the in the presence of poor carbon sources, such as oleate medium, the glucose sensing pathway is still inactive.

The addition of 2% of dextrose in oleate medium was enough to induce Atg36 phosphorylation in conditions where phosphorylation was absent before (Figure 7). However, this 2% dextrose, while sufficient to phosphorylate Atg36, is also enough to turn Tor1 on and inactivate the cell's autophagy machinery, preventing pexophagy from occurring. This led us to wonder if any other carbon sources that could phosphorylate Atg36 without also activating the Tor1 pathway (Figure 8). Every other carbon source tested was unable to initiate the glucose sensing pathway - Atg36 was not phosphorylated nor was Tor1 activated. These results show clearly that Tor1 inactivation alone, which occurred with most of these other carbon sources tested, is insufficient to trigger pexophagy – glucose is also necessary, likely to activate Atg36 via phosphorylation

(Figure 12). In addition to dextrose, it was found that only ethanol could activate Atg36 phosphorylation and pexophagy, but to a lesser extent.

The cell's response to either of these carbon sources was very rapid, by 15 minutes, their effects on both Atg36 phosphorylation and Tor1 activation had begun. However, the activation of Tor1 in nitrogen starvation conditions is transient. It is noteworthy that Atg36 phosphorylation precedes pexophagy, which happens only after Tor1 is subsequently inactivated over one to two hours (Figure 8). These results suggest that peroxisomes are first tagged for degradation via phosphorylation mediated by glucose, followed by engagement and activation of the autophagy machinery via Tor1 inactivation, stimulated by nitrogen starvation.

PKA phosphorylate S6 when cells were moved from a poor carbon source to glucose under nitrogen starvation conditions

Serendipity, while trying to understand more about PKA's role in Atg36 phosphorylation, we observed an interesting phenomenon in regards to its influence on S6 phosphorylation. S6 phosphorylation, presumed to be the normal target of Sch9, was phosphorylated by PKA rather than Sch9 when cells were transferred from oleate medium (Figures 9, 10 and 11). Normally, there is no S6 phosphorylation in oleate medium and traditionally when monitored in standard glucose medium, S6 phosphorylation is dependent on Sch9. However, when cells were transferred from oleate to glucose medium, S6 phosphorylation occurred only when PKA was not inactivated by 1NM-PP1.

The glucose and nitrogen sensing pathways and Atg36 phosphorylation

It is known that glucose is required to induce pexophagy. However, our data indicated that glucose is directly implicated in Atg36 phosphorylation and suggested that PKA and/or Sch9 is/are directly or indirectly involved in Atg36 phosphorylation. Individually, neither PKA nor Sch9 were solely responsible for Atg36 phosphorylation (Figures 9 and 10). However, inactivation of PKA and Sch9 together can delay or inhibit Atg36 phosphorylation (Figure 11).

We strongly believe that PKA must be the kinase implicated in Atg36 phosphylation, when glucose is added. However, similar to the glucose signaling pathway, probably Sch9 can substitute for the PKA when PKA is absent. Most probably because of this, results were inconclusive in determining if PKA itself is responsible for the delay in Atg36 phosphorylation.

When Sch9 and PKA were inhibited, we expected a complete block in Atg36 phosphorylation, not just a delay. But it could be that 100 uM of 1NM-PP1 was not enough to completely inhibit both PKA and Sch9, as see by some residual S6 phsophorylation (Figure 11) and even the slightest bit of leftover activity of either of these kinases might be enough to induce Atg36 phosphorylation.

This indicated crossover between the roles and effects of glucose and nitrogen in the cell, point to a crucial component for pexophagy. It has already been observed that PKA and Sch9 both inhibit the cell's autophagy machinery in the presence of glucose or nitrogen, respectively, and that their absence is crucial for autophagy to occur. However, glucose also has a known role in pexophagy. These seemingly contradictory findings can be explained when examining nitrogen starvation in the presence of glucose, the condition used to induce pexophagy. Glucose's rapid effect on the cell through the

glucose sensing pathway is transient and takes only a few minutes; shortly after the activation of PKA by cAMP, PKA activity induces a negative feedback loop and PKA is inactive once again (Williamson, 2009). In the presence of glucose and nitrogen, PKA will remain active but through activation of the nitrogen sensing pathway. Because pexophagy happens in the presence of glucose but in nitrogen starvation conditions, the spike in activity of PKA when glucose was added might be sufficient to induce Atg36 phosphorylation. Shorty thereafter, both PKA and Tor1 are inactive, leaving the core autophagy machinery free to collaborate with the selective factors for pexophagy. Therefore, it may be this rapid phosphorylation of Atg36 by PKA followed by PKA's inactivation that allows PKA to perform both, seemingly contradictory roles.

Looking forward

Although there is still much work to be done, this research has been a step towards understanding the roles of nitrogen and carbon in pexophagy. These results have showed pexophagy is dependent on both nitrogen starvation and the presence of glucose, for the activation of autophagy machinery and phosphorylation of Atg36, respectively. However, there needs to be further investigation of these pathways and their effects on pexophagy, especially the glucose sensing pathway. To further understand the effects of the glucose sensing pathway on Atg36 phosphorylation, it is possible to artificially manipulate the glucose sensing pathway by activating it without the presence of glucose (for example, by using a continuously active Ras2 kinase mutant) to observe its effects on Atg36 phosphorylation and pexophagy even in the absence of glucose. Additionally, the direct kinase of Atg36 has recently been found, the casein kinase I, Hrr25. It would be interesting to see the relationship between glucose and Hrr25 activity. The results

obtained in these experiments indicate that PKA and Sch9 play redundant roles in pexophagy, but they do not explain the entire process. To progress with this research, the other kinases involved in the *S. cerevisiae* glucose sensing pathway related to pexophagy and Atg36 phosphorylation must be identified.

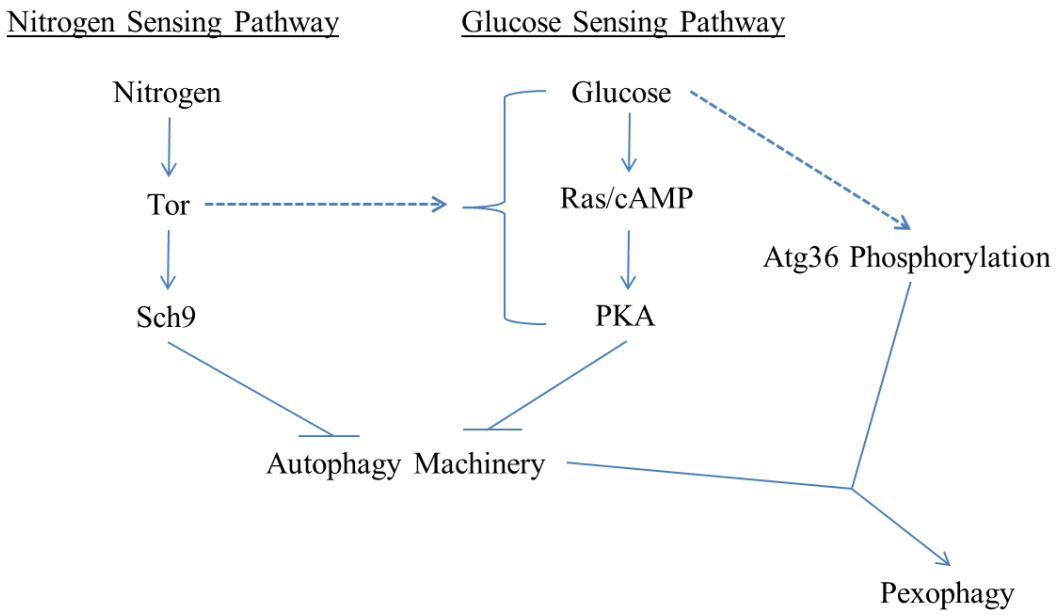

Figure 12. Signaling of autophagy in yeast. Extracellular nutrient levels influence autophagy in yeast by altering the activity of Tor1 and PKA. Tor1 is sensitive to nitrogen starvation while PKA and Ras are sensitive to glucose starvation.

IV:

Materials and Methods

Determining starvation conditions for various forms of autophagy

A uniform protocol was used to determine the conditions under which general autophagy, mitophagy, ER-phagy, and pexophagy occur. For each form of autophagy observed, wild type *S. cerevisiae* cells (BY4742) with a GFP tagged protein specific to the form of autophagy being studied was grown overnight in SD medium, YPL, or oleate respectively. After the strains reached between 1-3 OD, they were washed and split into nitrogen or carbon starvation conditions (SD-N or –D+N, respectively) at 1 OD/mL. This was considered time point 0 and all samples were left to rotate at 30^{o}C for six hours. 1 OD of cells were collected at time points 0, 2, 4 and 6 hours and immediately frozen at -80 ^{o}C. Following a TCA precipitation, all samples were used for a Western blot. Two Western blots were made per sample, one was screened with α-PS6 antibodies, the other with α-GFP antibodies which was subsequently stripped and screened for actin levels with α-Actin (from *S. cerevisiae*) antibodies as a loading control.

Atg36 phosphorylation in oleate medium in the presence of glucose

Jingjing Liu grew wild type *S. cerevisiae* yeast cells (BY4742) in SD overnight. After a sufficient growth, cells were washed and moved to oleate medium for two hours to induce peroxisome proliferation. Following this incubation, cells were washed again and moved either back to oleate or to oleate medium containing 2% glucose and rotated at 30^{o}C. This was considered time point 0 and at time points 0, 15, 30 and 60 minutes, 1 OD of cells were isolated and frozen in liquid nitrogen. After these samples were TCA precipitated, and run in a Western blot, which was screened for HA-Atg36 phosphorylation, S6 phosphorylation, Pot1-GFP cleavage, and F1β with α-HA antibodies, α-PS6 antibodies, α-GFP antibodies, and α-F1β antibodies, respectively.

Atg36 phosphorylation in various carbon sources

Jingjing Liu grew wild type *S. cerevisiae* yeast cells (BY4742) in SD overnight. Once cells grew to a sufficient level, they were washed and incubated in oleate medium for two hours while rotating at 30^{o}C to induce peroxisome induction. After two hours, cells were washed again and moved to one of 6 different starvation conditions at 1 OD/mL; nitrogen starvation with 2% glucose (+D-N), nitrogen starvation with 2deoxyglucose (2DG-N), nitrogen starvation with glycerol (Gly-N), nitrogen starvation with ethanol (Eth-N), nitrogen starvation with galactose (Gal-N), and both carbon and nitrogen starvation (-D-N). Each of these samples were rotated at 30^{o}C for two hours as time points at 0, 15, 30, 60 and 120 minutes were obtained by isolating 1OD of cells and immediately freezing them with liquid nitrogen. Following a TCA precipitation, they were run in a Western blot, which was screened for HA-Atg36 phosphorylation, S6 phosphorylation, Pot1-GFP cleavage, and F1β with α-HA antibodies, α-PS6 antibodies, αGFP antibodies, and α-F1β antibodies, respectively.

Effects of PKA and Sch9 inactivation on Atg36 phosphorylation

PKA and Sch9 are both proteins crucial to cell growth, thus, analog sensitive mutants were used since traditional mutation methods were impossible. The Sch9 analog sensitive mutant and the Sch9/PKA analog sensitive double mutant were in one parental background, while the PKA analog sensitive mutant was in another. All five strains were grown in their selective SD medium overnight. When they reached 1 OD/ml, they were washed and grown in their selective oleate medium at 1 OD/mL to induce peroxisomes. After rotating at 30^{o}C for two hours in oleate medium, the cells were washed and moved to carbon and nitrogen starvation conditions at 2OD/mL. A 10mM stock solution of

1NM-PP1, the drug used to induce the analog sensitive mutations in PKA, Sch9 or both, was added to each sample until it was diluted to 100uM. Cells were left to incubate with the drug for one hour while rotating at 30^{o}C. After the drug was given sufficient time to permeate all of the cells and inactivate its target(s), dextrose was added to the –D-N solution to bring it up to 2% of dextrose. The addition of dextrose marked time point 0. 2OD/mL of cells were left to rotate in this 2% SD-N solution containing 100uM 1NM-PP1 at 30^{o}C as five time points were obtained. At 0, 8, 15, 30 and 60 minutes, 1OD of cells were collected and immediately frozen in liquid nitrogen. Following a TCA precipitation, all samples were run through a Western blot. Each blot was screened for PS6, HA-Atg36, and F1β with the use of α-HA antibodies, α-PS6 antibodies, and α-F1β antibodies, respectively.

Table 1. Yeast strains used for autophagy assays

Strain	Description	Reference
BY4742	Wild type	
OM45-GFP	OM45-GFP	J. Wilhelm GFP library
Sec61-GFP	Sec61-GFP	J. Wilhelm GFP library
GFP-Atg8	GFP-Atg8	Helen Shang
Pot1-GFP	Pot1-GFP	Jingjing Liu

Table 2. Yeast strains used for Atg36 phosphorylation assays

Strain	Description	Reference
BY4742	Wild type	
STY 117	PKA*	This study
STY117W	Wild type for STY117	This study
STY121	Sch9*/PKA*	This study
STY116	Sch9*	This study
STY116	Wild type for STY137 and STY116	This study

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