The regulation of Atg1 protein kinase activity is important to the autophagy process in

*Saccharomyces cerevisiae*

**Dissertation**

Presented in Partial Fulfillment of the Requirements for the Degree
Doctor of Philosophy in the Graduate School of The Ohio State University

By

Yuh-Ying Yeh

Graduate Program in Molecular, Cellular and Developmental Biology

The Ohio State University

2010

Dissertation Committee:

Paul K Herman, Adviser

Stephen A Osmani

Amanda A Simcox

Mark R Parthun
Abstract

Autophagy is an evolutionarily conserved, degradative pathway that has been implicated in a number of physiological processes such as development and aging as well as cancer and innate immunity. This pathway is important for cell survival in starvation and is considered as a potential target for therapeutic intervention in a number of pathological conditions. Therefore, it is important that we develop a thorough understanding of the mechanisms regulating this trafficking pathway. Autophagy was initially identified as a cellular response to nutrient deprivation and is essential for cell survival during periods of starvation. During autophagy, an isolation membrane emanates from a nucleation site that is known as the phagophore assembly site (PAS). This membrane encapsulates nearby cytoplasm to form an autophagosome that is ultimately targeted to the vacuole/lysosome for degradation. The small molecules produced are then recycled and used by cells during this period of starvation. Autophagy activity is highly regulated and multiple signaling pathways are known to target a complex of proteins that contains the Atg1 protein kinase.

Atg1 protein kinase activity is essential for normal autophagy in all eukaryotes and appears to be controlled tightly by a number of kinases, which target this enzyme and its associated protein partners. Our data and that of others have established that Atg1 activity is regulated, at least in part, by protein phosphorylation. In this work, we identified a particular phosphorylation event on Atg1 as an important control point within
the autophagy pathway in *Saccharomyces cerevisiae*. This phosphorylation occurs at a threonine residue, T226, within the Atg1 activation loop that is conserved in all Atg1 orthologs. This activation loop phosphorylation is essential for Atg1 kinase activity and the induction of autophagy. The data also suggested that promoting this autophosphorylation is a primary role for two key conserved regulators of Atg1 activity, Atg13 and Atg17. Atg13, in particular, appears to stimulate this phosphorylation by promoting an Atg1 self-interaction. In all, these data suggest that autophosphorylation within the Atg1 activation loop may represent a point of regulatory control for this degradative process.

To further our knowledge of phosphorylation in Atg1, we used a combined mass spectrometry and molecular biology approach to identify and characterize additional sites of phosphorylation in Atg1. Fifteen sites of phosphorylation were discovered here, including nine that had not been noted previously. Alterations of these sites identified a number of positions that appear to be important for full autophagy activity *in vivo*. One site was of special interest as it was within a highly conserved motif, the Gly-rich loop, in the Atg1 kinase domain. Phosphorylation at this equivalent position inhibits the kinase activity of particular cyclin-dependent kinases and we showed here that this site may serve a similar function in Atg1. In addition, we identified Ser-390 as the site of autophosphorylation responsible for the anomalous migration observed for Atg1 on SDS-polyacrylamide gels. In all, the analyses here identified a number of potential sites of regulation in the Atg1 protein that provide important insight into the control of the autophagy process and will form a framework for future studies with this enzyme.
Dedication

This work is dedicated to my family for their love and support in this journey.
Acknowledgments

I am very grateful to my adviser Dr. Paul Herman for being a mentor in the truest sense throughout my graduate career. I appreciate his guidance and intellectual support that have made this dissertation possible. His passion for science has motivated me to become a creative and critical scientist. I would also like to thank my committee Dr. Stephen Osmani, Dr. Amanda Simcox, Dr. Mark Parthun and Dr. Berl Oakley for their encouragement, valuable input and generous help in my graduate study and career development.

I would like to thank Joe Stephan to help me start my project in the very beginning and Vidhya Ramachandran to support me throughout my Ph.D. life. I enjoy their company in the lab and the brilliant ideas every Friday afternoon. I also thank Khyati Shah, Kristie Wrasman and Demetra Stamatakos to assist my research projects and all the current and former members of the great Herman lab for sharing their knowledge and resources to me.

I also wish to thank Dr. Yoshinori Ohsumi, Dr. Yoshiaki Kamada, Dr. Daniel Klionsky, Dr. Jeremy Thorner, Dr. Michael Snyder, Dr. Helen Chamberlin, Dr. Russell Hill, Dr. Anita Hopper, Dr. Harold Fisk and Dr. Hui-Lin Liu for providing valuable reagents and equipment used in this study and our collaborators, Dr. Kay-Hooi Khoo, Dr.
He-Hsuan Hsiao and Dr. Chi-Chi Chou for mass spectrometry analysis in the Core Facility for Proteomics, Academia Sinica, Taiwan.

I would also like to thank friends I met in Columbus and Taiwan for their friendship and support in the past few years. Finally, I would like to thank my parents, sister and my husband, Chi-Yueh Kao for their love, patience and faith in me.
Vita

May 1981 .......................................................Born – Hualien, Taiwan

1999 – 2003....................................................B.S. Applied Chemistry, National Chaio-
Tung University, Taiwan

2004 to present ..............................................Graduate Research Associate, Department
of Molecular Genetics, The Ohio State
University

Publications

D., Khoo K.H. & Herman P.K. The identification and analysis of phosphorylation sites
on the Atg1 protein kinase. *Autophagy* (In revision).

Yeh Y.Y., Wrasman K., & Herman P.K. Autophosphorylation within the Atg1 activation
loop is required for both kinase activity and the induction of autophagy in *Saccharomyces

Stephan J.S., Yeh Y.Y., Ramachandran V., Deminoff S.J., & Herman P.K. The Tor and
cAMP-dependent protein kinase signaling pathways coordinately control autophagy in *Saccharomyces

Stephan J.S., Yeh Y.Y., Ramachandran V., Deminoff S.J., & Herman P.K. The Tor and
PKA signaling pathways independently target the Atg1/Atg13 protein kinase complex to

Chen Y.Y., Lin S.Y., Yeh Y.Y., Hsiao H.H., Wu C.Y., Chen S.T., & Wang A.H. A
modified protein precipitation procedure for efficient removal of albumin from serum.

**Fields of Study**

Major Field: Molecular, Cellular and Developmental Biology
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Chapter 1

Introduction

The term ‘autophagy’ is derived from the Greek words ‘auto’ meaning self, and ‘phagy’ meaning eat. Autophagy is an evolutionarily conserved process in eukaryotes by which cytoplasmic cargo sequestered inside double-membrane vesicles are delivered to the lysosome for degradation. This ‘self-eating’ process not only mediates turnover of misfolded proteins and damaged organelles but also is a cellular response to supply nutrients and energy in response to various stresses. Autophagy has been connected to human pathophysiology, and research on this pathway has had implications for cancer, neurodegeneration, immune responses, development and ageing.

Macroautophagy (hereafter referred to as autophagy) is an intracellular degradation system that delivers the cytoplasmic components and organelles of a cell to lysosomes for degradation. The induction of autophagy leads to de novo formation of cup-shaped membranes in the cytoplasm termed phagophores, which expand and engulf portions of the cytoplasm (Figure 1.1). This leads to the formation of double-membrane-bound, sequestering vesicles, called autophagosomes. The outer membrane of an autophagosome subsequently fuses with lysosomes, exposing the inner compartment to lysosomal hydrolases. Eventually the inner membrane of the autophagosome, together with the enclosed cargo, is degraded, and the resulting
macromolecules are released into the cytosol for recycling. Therefore, in contrast to the ubiquitin–26S proteasome system, autophagy mediates primarily non-selective and bulk degradation of many intracellular proteins and organelles.

Accumulating evidence reveals that autophagy is linked to various human diseases. Certain aggregate-prone proteins, such as those involved in Huntington’s disease, were found to depend on autophagy for their clearance. Cancer was also found to be genetically linked to impaired autophagy. In addition, research studies using neuronal-specific knockout mice, demonstrated that basal autophagy controlled the turnover of cytosolic proteins to prevent the accumulation of abnormal neuroproteins that may cause neurodegeneration. Other work also suggests that autophagy is involved in the defense mechanism against certain invading bacterial pathogens and viruses.

1.1 Non-selective versus selective autophagy

In addition to autophagy, the yeast *Saccharomyces cerevisiae* utilizes a similar system named the cytoplasm-to-vacuole targeting (Cvt) pathway, in which Cvt vesicles transport two vacuolar enzymes, aminopeptidase I and α-mannosidase, to the vacuole. However, there are several major differences between autophagy and the Cvt pathway. The Cvt pathway constitutively functions under nutrient rich conditions, while autophagy is actively elevated during starvation. In contrast to the highly selective Cvt pathway, autophagy is largely nonselective. In addition, Cvt vesicles (150–250 nm diam) are smaller than autophagosomes (400–900 nm diam). Importantly, the Cvt pathway has only been described in *S. cerevisiae* and closely related fungal species.
Although the Cvt pathway and other types of autophagy, like pexophagy, mitophagy and macroautophagy, are morphologically and mechanistically similar, they are different in important ways.\textsuperscript{28-30} Pexophagy, mitophagy and nonspecific macroautophagy are degradative, whereas the Cvt pathway is biosynthetic, delivering at least two resident hydrolases to the vacuole.\textsuperscript{31,32} More, macroautophagy mediates bulk degradation of cytoplasmic constituents whereas mitophagy and pexophagy specifically degrade damaged mitochondria and peroxisomes. Overall, they share one subset of the Atg proteins that are essential for autophagosome formation and referred to as the core molecular machinery.\textsuperscript{5}

1.2 \textbf{Autophagy-related, ATG genes}

To date, 34 \textit{ATG} (AuTophagy-related) genes have been reported in \textit{Saccharomyces cerevisiae} and other fungi. \textit{ATG} genes are evolutionally conserved, and most of the \textit{ATG} genes have been identified in higher eukaryotes.\textsuperscript{33,34} Fifteen of these genes are essential for both macroautophagy and selective autophagy and are categorized as part of the core autophagic machinery.\textsuperscript{5} The core machinery encodes the fundamental proteins for the biogenesis of autophagy-related membranes. Characterization of these 15 \textit{ATG} proteins revealed five major functional groups: (I) Atg1 and its regulators,\textsuperscript{35} (II) the autophagy-specific phosphatidylinositol (PtdIns) 3-kinase complex, (III) Atg8 conjugation system, (IV) Atg12 conjugation system,\textsuperscript{37} and (V) a subgroup of functionally unknown proteins that interact with each other (Figure 1.2).\textsuperscript{38-41} Furthermore, in yeast the autophagy machinery is concentrated at a peri-vacuolar (the vacuole is the yeast equivalent of the lysosome) site termed the phagophore assembly site (PAS), and the concerted action of
the autophagy machinery at the PAS leads to phagophore expansion and autophagosome formation.42,43.

In addition to the core machinery, Atg proteins required for macroautophagy can be classified into five functional groups: (I) the Atg1 kinase complex (Atg1-13-17-29-31), (II) the class III phosphatidylinositol (PI)3–kinase complex (Atg6–Atg14–Vps15–Vps34), (III) the Atg12 conjugation system (Atg12–Atg5–Atg16), (IV) the Atg8 conjugation system (Atg8–PE) 44 and (V) the PI(3)P-binding Atg2–Atg18 complex and Atg9 (Table 1.1). A comprehensive hierarchical analysis revealed that Atg1 complex activation is the most initial step of autophagosome formation 45. Therefore, a complete understanding of the role of the Atg1 complex is essential for the development of the autophagy regulation models.

1.3 The molecular machinery of autophagy in yeast

Induction

In the yeast Saccharomyces cerevisiae, basal-level autophagy is very low under nutrient-rich conditions. However, upon nutrient deprivation and other extracellular cues, autophagy is efficiently induced. A central inhibitor of autophagy is the serine/threonine protein kinase Tor (Target of rapamycin). Tor integrates input signals from multiple upstream signal transduction pathways and negatively regulates the Atg1 protein complex in nutrient-rich conditions.35,46-48 (Figure 1.3). When Tor is inhibited by starvation or rapamycin treatment, the kinase activity of Atg1 is activated and the binding affinity among Atg1, Atg13 and Atg17 is also increased.35 These interactions promote the formation of an Atg1-Atg13-Atg17 scaffold and the subsequent recruitment of multiple
Atg proteins to the PAS to initiate autophagosome formation\textsuperscript{45,49-52}. Thus, a role of the Atg1 kinase complex in protein recruitment is indispensable for autophagy induction.

**Autophagosome formation**

Double-membrane autophagosomes are thought to be assembled at the PAS by the addition of new membrane; however, the origin of this membrane is unclear. Previous reports have suggested mitochondria, the Golgi complex, or the ER as the membrane source\textsuperscript{53-55}. Under nutrient-rich conditions, the Atg proteins are dispersed in the cytoplasm and assemble into a perivacuolar spot in response to nutrient starvation\textsuperscript{56}. Deletion of either Atg1, Atg13, Atg17, Atg29 or Atg31 completely abolishes spot formation of all other core Atg proteins, including that the Atg1 protein complex functions together with the Ag17-Atg29-Atg31 complex to organize the PAS for autophagosome formation\textsuperscript{50,52}. Therefore, multiple Atg proteins are recruited in a highly orchestrated manner to the phagophore to participate in autophagosome biogenesis (Figure 1.4).

The nucleation and assembly of the phagophore membrane in autophagy require the class III phosphatidylinositol 3-kinase (PtdIns3K) complex I, which is composed of the PtdIns 3-kinase Vps34 (vacuolar protein sorting 34), a myristoylated serine/threonine kinase Vps15 (p150 in mammalian cells), Atg14 (BARKOR or mAtg14 in mammalian cells) and Atg6/Vps30 (Beclin 1 in mammalian cells)\textsuperscript{9,34,57,58}. The PtdIns3K complex produces PtdIns3P (phosphatidylinositol 3-phosphate), which is used to recruit PtdIns3P-binding proteins, like Atg18\textsuperscript{59}). Atg18 forms a complex with Atg2 and functions in autophagosome formation while the PtdIns3P-Atg18 interaction is important for
localization of this complex to the autophagic membrane\textsuperscript{45,59}.

The PtdIns3K complex I, in part together with the above Atg proteins, further recruits two ubiquitin-like (Ubl) conjugation systems, Atg12–Atg5–Atg16 and Atg8–PE (phosphatidylethanolamine), to the phagophore (Figure 1.4)\textsuperscript{43,45}. Both conjugates localize to autophagy-related membranes, suggesting their direct involvement in regulating the membrane elongation and expansion of the forming autophagosome (Figure 1.5, C). In the Atg8 conjugation system, Atg8 is first processed by a cysteine protease, Atg4, exposing a C-terminal glycine residue (Figure 1.5, A). The E1 activating enzyme, Atg7, activates Atg8 to form the intermediate and then transfers Atg8 to Atg3 (E2 conjugating enzyme). Atg8 eventually forms an amino bond with the target lipid PE and in this form, localizes to the isolation membrane and the autophagosome\textsuperscript{60}.

Atg12 undergoes conjugation in a similar manner as the Atg8-PE system (Figure 1.5, B). Atg12 is activated by the common E1 enzyme, Atg7, transferred to a specific E2 enzyme, Atg10, and forms an isopeptide bond to the substrate protein, Atg5. The Atg12–Atg5 conjugate further interacts with self-oligomerized Atg16 to form the Atg12–Atg5–Atg16 complex that then attaches to the phagophore\textsuperscript{61,62}. In contrast to ubiquitination, Atg12–Atg5 conjugation is constitutive and irreversible, and no substrate-specific E3 ligase counterpart is apparently required in this process\textsuperscript{63}. The involvement of the Atg12–Atg5–Atg16 complex in the Atg8–PE system has been implied genetically, as mutations that abolish the former complex significantly decrease the production of Atg8–PE\textsuperscript{43,64}. It has also been shown that purified Atg12–Atg5 conjugates can stimulate the formation of Atg8–PE \textit{in vitro}\textsuperscript{65,66}, where Atg12–Atg5 directly interacts with the E2
enzyme, Atg3 to enhance its activity (Figure 1.5, A–B). Thus, Atg12–Atg5 is proposed to have an E3-like function in the Atg8 lipidation.

Atg8 is predominantly cytosolic in nutrient-rich conditions but upon autophagy induction, this protein is conjugated to PE and is localized to the PAS \(^{60,67}\). Therefore, the lipidation of Atg8 and its mammalian homolog, LC3, is widely used to monitor autophagy induction.

**Degradation and recycling**

When autophagosome formation is completed, autophagosomes fuse with lysosomes (in metazoan cells) or vacuoles (in yeast and plant cells). These fused compartments are often called “autolysosomes” or “autophagolysosomes.” Degradation of the inner membrane and the cellular contents of autophagosomes is dependent on a series of lysosomal/vacuolar hydrolases, including proteinases A and B and the lipase Atg15 \(^{27}\). Atg15, a putative lipase is involved in the turnover of autophagic bodies \(^{68,69}\). Once macromolecules have been degraded in the lysosome/vacuole, the resulting small molecules are exported back to the cytosol for reuse. However, little is known about this step. In yeast Atg22, a membrane protein required for the breakdown of autophagic bodies, has been identified as a putative amino acid efflux pump that can recycle amino acids from the vacuole to cytosol with other vacuolar permeases, such as Avt3 and Avt4 \(^{70,71}\).

**1.4 Signaling regulation of autophagy**
Autophagy in yeast is mainly a response to nutrient starvation. Tor, a phosphatidylinositol kinase-related protein kinase, is involved in the induction of autophagy since the inhibition of Tor by rapamycin mimics starvation and induces autophagy, even under nutrient-rich conditions. The addition of cyclic AMP suppresses the induction of autophagy by nutrient starvation, suggesting that the cAMP-dependent protein kinase, PKA, also has an inhibitory role in autophagy. In contrast, Snf1 the yeast homolog of the AMP-activated protein kinase, is suggested to be a positive regulator of autophagy.

**Tor complex 1**

The Tor protein forms two distinct complexes, Tor complex 1 (TORC1) and TORC2. Only TORC1 function is sensitive to rapamycin, indicating that TORC1 is responsible for controlling autophagy. The Tor signaling pathway regulates the induction phase of autophagy through the Atg1 protein complex. This is accomplished through the Tor-dependent hyperphosphorylation of Atg13, which prevents the Atg1-Atg13 association and hence, Atg1 kinase activation. Upon inactivation of Tor by rapamycin, Atg13 is rapidly dephosphorylated, leading to the activation of Atg1 and the induction of autophagy pathway.

**The Ras/cAMP-dependent protein kinase (PKA)**

The Ras/PKA signaling pathway plays an important role in glucose sensing from yeast to mammals. Yeast PKA is a heterotetramer composed of two of the regulatory subunits Bcy1, and three of the catalytic subunits Tpk1, Tpk2, and Tpk3. In nutrient-rich...
conditions, the small GTPases, Ras1 and Ras2, are active and enhance cAMP production by the adenylyl cyclase. This cAMP binds to Bcy1 and releases its inhibition of the catalytic subunits. Constitutive activation of the Ras/PKA pathway suppresses the autophagy induced by rapamycin in yeast \(^{79,80}\), suggesting that the Ras/PKA pathway downregulates autophagy in parallel with the TOR pathway. Autophagy inhibition by Ras/PKA is also mediated through the Atg1 protein complex \(^{73,74}\). In the presence of nutrients, PKA phosphorylation causes the cytosolic distribution of Atg1 and Atg13, whereas during starvation, Atg1 and Ag13 are dephosphorylated and localized to the PAS independently. In addition to PKA, the protein kinase, Sch9, a homolog to the mammalian ribosomal S6 kinase (S6K), is involved in nutrient sensing \(^{73,81,82}\).

**AMP-activated protein kinase (AMPK)**

During starvation, activation of autophagy is essential for cell survival. In mammalian cells, increases in the cellular AMP:ATP ratio are sensed by the AMP-activated protein kinase (AMPK) and the active AMPK can indirectly inhibit the TORC1 complex to induce autophagy \(^{83-85}\). Genetic evidence from yeast suggests that the yeast Snf1 protein kinase complex, the homolog of the mammalian AMPK, can positively modulate autophagy through the Atg1 protein complex \(^{39}\).

**1.5 The Atg1 protein complex – Atg1 protein kinase and its regulators**

Atg1, a Ser/Thr protein kinase, contains a conserved kinase domain at its N-terminus and its activity is essential for autophagy. Atg1 kinase activity is greatly enhanced following nutrient starvation or the addition of rapamycin \(^{35,86}\). In yeast, Atg1 associates with at
least eight other Atg proteins, Atg13, 17, 29, 31, 11, 20, 24, and Vac8. The Atg1 kinase and its regulators, Atg13, Atg17, Atg29, and Atg31, collaboratively function in the initial step of autophagosome formation. These proteins comprise the most upstream Atg subfamily in the hierarchy of the localization of Atg proteins to the PAS. The regulation of Atg1 kinase activity involves Atg13 and Atg17. Atg13 is phosphorylated in a TORC1-dependent manner under nutrient-rich conditions and it is rapidly dephosphorylated in response to starvation or inactivation of TOC1 by rapamycin treatment. Dephosphorylated Atg13 associates with Atg1 and leads to upregulation of Atg1 kinase activity. However, the precise mechanism by which Atg13 promotes Atg1 kinase activity is not yet clear. Phosphorylation of one or more factors by Atg1 is expected to trigger a downstream event in autophagosome formation and several Atg proteins are phosphorylated in an Atg1-dependent manner both in vivo and in vitro. Nevertheless, the physiological relevant substrates of Atg1 still remain elusive.

While Atg1 and Atg13 are in the core machinery of autophagosome formation, Atg17, Atg29, and Atg31 are more specifically required for starvation-induced autophagy (Figure 1.2). These latter three proteins constitutively exist as a ternary complex that associates with the Atg1–Atg13 complex and stimulates Atg1 activity in response to nutrient starvation. This association is also a prerequisite for the recruitment of other core Atg proteins to the PAS during the induction of autophagy, suggesting that this complex functions as a nucleation site for the autophagosome biogenesis. Although Atg1 kinase activity is required for autophagy, it is dispensable for the formation of the Atg1-Atg13-Atg17-Atg29-Atg31 complex and the recruitment of these proteins to the PAS. However, the release of Atg8 from the PAS to
autophagosome requires the kinase activity of Atg1\textsuperscript{50,52,94}. In all, these studies suggest that the kinase activity of Atg1 is involved in the dynamics of the Atg proteins at the PAS.

1.6 Regulation of the Atg1 protein complex

Tor and PKA are master regulators of nutrient signaling and negative regulators of autophagy through the Atg1 protein complex\textsuperscript{47,72,95,96}. Rapamycin, an inhibitor of TORC1 mimics starvation and activates autophagy even under nutrient rich conditions. Rapamycin treatment rapidly induces Atg13 dephosphorylation and hence promotes Atg1–Atg13–Atg17 complex formation and activation of Atg1\textsuperscript{35,51}. Although it was proposed that dephosphorylation of Atg13 is one of the initial events in autophagy, whether Atg13 is a direct target of TORC1 or whether dephosphorylation of Atg13 is sufficient for the induction of autophagy is not clear. However, it has recently been shown that an unphosphorylatable Atg13 mutant can at least partially induce autophagy in non-starved cells and that Atg13 can be directly phosphorylated by TORC1 in vitro at multiple serine residues\textsuperscript{92}. In addition to TORC1, PKA inhibits autophagy through the direct phosphorylation of Atg1 and Atg13\textsuperscript{73}. Atg1 and Atg13 variants that contained Ser-to-Ala mutations of the PKA sites were constitutively localized to the PAS regardless of PKA activity or nutrient conditions. These results suggested that the PKA signaling might also regulate the initial step of autophagosome formation. The inhibitory effects of TORC1 and PKA are independent and additive, and the sites of TORC1 and PKA phosphorylation on Atg13 likely differ\textsuperscript{73}. 
This TORC1–Atg1/mTOR–ULK1 signaling module has been shown to be conserved among most eukaryotes to regulate autophagy [46,97-101]. For example, several recent studies in mammals and Drosophila identified a direct interaction between the mTOR and the ULK1 complexes [98,99,101,102]. Similar to yeast, mTOR forms two distinct complexes termed mTORC1 and mTORC2, where mTORC1 is highly sensitive to cellular nutrient conditions and rapamycin [76,103]. Interestingly, mTORC1, but not mTORC2, is incorporated into the large ULK1–mAtg13–FIP200 complex and its recruitment is independent of mAtg13 [104]. Both in vitro and in vivo data suggest that mTORC1 phosphorylates ULK1 and mAtg13 under nutrient-rich conditions and ULK1 phosphorylates mAtg13 and FIP-200 [99,104,105]. In Drosophila, dTor also interacts with dAtg1 and phosphorylates dAtg1 and dAtg13 in a nutrient-dependent manner [46]. Interestingly, Atg1/Atg13 can conversely regulate the dTor pathway, which likely serves as a negative feedback loop in Drosophila [46,47,106]. However, the molecular mechanism of mTORC1 regulation of the kinase activity of dAtg1/ULK1 remains unknown and additional work is needed in order to identify the TORC1 phosphorylation sites present in the dAtg1/ULK1.

1.7 The Atg1 complex in higher eukaryotes

The ATG genes required for autophagy are evolutionally conserved, and many of the mammalian ATG genes have been identified by conventional protein sequence searches [33,34,107]. Atg1 is highly conserved among eukaryotes (Table 1.2), and the mammalian homologs of Atg1 are the unc-51-like kinase 1 (ULK1) and 2 [108,109]. ULK1 and ULK2 were first identified as homologs of Caenorhabditis elegans uncoordinated-51 (unc-51)
and the role of ULK1 in autophagy was later characterized \(^{97,100,110,111}\), whereas the function of ULK2 is less clear. ULK1-deficient mice have overall normal development, suggesting that ULK2 might be partially redundant \(^{112}\). Nonetheless, silencing of ULK1 alone is sufficient to block autophagy, suggesting that ULK1 may be the major homolog needed for this process \(^{97,110}\). Atg1/ULK1 orthologs have been found in many other species, including *S. pombe*, *Dictyostelium discoideum*, *C. elegans* (known as Unc-51), and *D. melanogaster*, and all have clear roles in autophagy \(^{47}\). In addition to ULK1 and ULK2, there are three related proteins in mammals: ULK3, ULK4, and STK36 \(^{113,114}\). These proteins are less conserved and have not been well studied \(^{97,99-101}\).

The ULK1-binding partners required for autophagy recently have been discovered. The ULK1 complex includes at least three autophagy-related proteins, mammalian Atg13 \(^{46,98,99,101}\), FIP200 (focal adhesion kinase family interacting protein of 200 kD) \(^{100}\), and Atg101 \(^{102,104}\). FIP200, which is considered a functional ortholog of Atg17, is a multifunctional protein and plays roles in the control of cell adhesion, migration, proliferation, cell size, and cell death \(^{115,116}\). Atg101 is a novel small protein, which associates with the ULK protein complex through mAtg13. Atg13 homologs are also conserved from yeast to most eukaryotes (Table 1.3) \(^{46,97}\). However, FIP200 and Atg101 are not found in the yeast *S. cerevisiae*, but conserved homologs can be identified in *C. elegans* and *D. melanogaster*. Moreover, homologs of Atg29, and Atg31 have not yet been identified in *C. elegans*, *D. melanogaster*, and mammals.

The cellular distribution of the mammalian ULK protein complex is regulated in a similar fashion as the yeast Atg1 protein complex. ULK1, mAtg13, FIP200, and Atg101 are predominantly localized to the cytosol and associate with the isolation membrane
upon autophagy induction \(^{97,98,100-102,104,111}\). Like yeast, this protein complex is the most upstream unit in the hierarchical relationship among all Atg proteins localized to the autophagosome formation site and is essential for autophagosome formation \(^{116}\). Several studies have shown that the interaction of ULK1-mAtg13-FIP200 is regulated similarly as their yeast counterparts \(^{99,101}\). Thus, the assembly of the Atg1/ULK1 protein complex seems to be regulatory event in autophagy among all species.

1.8 Concluding remarks

Nutritional deprivation was likely a frequent and crucial stress encountered by the earliest eukaryotes. Therefore, a starvation-induced mode of autophagy, which is essential for cell survival, would have been established early and likely conserved during evolution. In fact, homologs of proteins of the core machinery have been identified in all eukaryotes, and most of them participate in autophagy. Recent studies, especially in higher eukaryotes such as mammals, have rapidly unveiled the diversity and complexity of autophagy. Investigations of the Atg1/ULK1 complex have revealed its roles in nutrient sensing, the assembly of autophagosome, and the regulation of autophagy that have been well conserved from yeast to mammals. Moreover, these studies also highlighted additional functions and regulatory mechanisms of the Atg1/ULK1 complex in higher eukaryotes, and a number of diverse constituents of the Atg1/ULK1 complex were found. Thus, the function of the Atg1/ULK1 complex in higher eukaryotes has been apparently expanded during evolution and it is a key cellular regulator in these eukaryotes. An in-depth knowledge of the diverse modes of autophagy is important for an accurate
understanding of the molecular mechanism in each physiological condition that might shed light on medical applications.

Our current knowledge of the organization and functions of the core machinery is still quite limited, in part because very few of the Atg proteins have clear functional motifs. Accordingly, continued efforts are needed to elucidate the fundamental steps of autophagosome biogenesis, including the origin of membrane used to form this intermediate. A better understanding of the relationships among the Atg1/ULK1 protein complex components is required to fully understand the regulation of Atg1/ULK1 kinase activity. Moreover, the identification of Atg1/ULK1 substrates and other binding partners is essential to enhance our knowledge of the precise role of this kinase in autophagy. Finally, there are differences between starvation-induced and other types of autophagy. However, an extended understanding of the specific adaptations that allow the autophagy machinery to meet other physiological circumstances can only be achieved once we have uncovered the basic autophagy system.
<table>
<thead>
<tr>
<th>Subgroup components</th>
<th>Known or putative function</th>
<th>References</th>
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</table>
| **Atg1 kinase and its regulators**
Atg1 | Ser/Thr kinase | 35, 50, 52, 86, 118 |
Atg13 | TORC1 substrate involved in the regulation of Atg1 activity | 35, 50, 52, 78, 86, 118 |
Atg17 | Forms a starvation-induced autophagy-specific complex with Atg29 and Atg31, which further associates with Atg1 and Atg13 | 45, 49, 94 |
Atg29 | Component of the Atg17–Atg29–Atg31 complex | 52, 91 |
Atg31 | Component of the Atg17–Atg29–Atg31 complex | 52, 90 |
| **PtdIns 3-kinase complex**
Vps34 | PtdIns 3-kinase | 57, 119 |
Vps15 | Ser/Thr kinase required for Vps34 activity | 57, 119 |
Vps30/Atg6 | Component of unknown function | 57, 119 |
Atg14 | Recruits the PtdIns 3-kinase complex to the PAS | 57, 119 |
| **Atg12 conjugation system**
Atg12 | Ubiquitin-like protein that is conjugated to Atg5 | 37, 64, 65 |
Atg5 | A target of Atg12 and interacts with Atg16 | 37, 64, 65 |
Atg7 | Common E1 enzyme for Atg12–Atg5 and Atg8–PE formation | 37 |
Atg10 | Specific E2 enzyme for Atg12–Atg5 formation | 37, 61, 120 |
Atg16 | Required for the PAS localization of Atg12–Atg5 | 61 |

Table 1.1 Subgroups of Atg proteins required for starvation-induced autophagy. Copyright © 2009 Nature Publishing Group. *Trigger and regulate PAS assembly of other proteins. ‡Produces PtdIns3P at the PAS. §Stimulates and determines the location of Atg8 lipidation. || Controls membrane tethering and hemifusion. ¶The function of this complex is unknown. PAS, pre-autophagosomal structure; PE, phosphatidylethanolamine; PtdIns, phosphatidylinositol; TORC1, target of rapamycin complex 1; Vps, vacuolar protein sorting (Continued to p17).
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<th>Subgroup components</th>
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<tr>
<td><strong>Atg8 conjugation system</strong>*</td>
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<tr>
<td>Atg8</td>
<td>Ubiquitin-like protein conjugated to PE</td>
<td>36,60,121-126</td>
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<tr>
<td>Atg3</td>
<td>Specific E2 enzyme for Atg8–PE formation</td>
<td>36,121,124</td>
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<td>Atg4</td>
<td>Removes the carboxy-terminal Arg and conjugated PE from Atg8 62–64</td>
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<td>Atg7</td>
<td>Common E1 enzyme for Atg12–Atg5 and Atg8–PE formation</td>
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<td><strong>Atg2–Atg18 complex¶ and Atg9</strong></td>
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<tr>
<td>Atg2</td>
<td>Forms a complex with Atg18, which is involved in Atg9 dynamics at the PAS</td>
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<tr>
<td>Atg18</td>
<td>Binds to PtdIns3P</td>
<td>59,128,129</td>
</tr>
<tr>
<td>Atg9</td>
<td>Integral membrane protein of unknown function</td>
<td>40,94,118,130</td>
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Table 1.1 Subgroups of Atg proteins required for starvation-induced autophagy. Copyright © 2009 Nature Publishing Group. *Trigger and regulate PAS assembly of other proteins. ‡Produces PtdIns3P at the PAS. §Stimulates and determines the location of Atg8 lipidation. ¶Controls membrane tethering and hemifusion. || Controls membrane tethering and hemifusion. †The function of this complex is unknown. PAS, pre-autophagosomal structure; PE, phosphatidylethanolamine; PtdIns, phosphatidylinositol; TORC1, target of rapamycin complex 1; Vps, vacuolar protein sorting.
<table>
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<td>Taz1IF1?</td>
<td>NP_509241.1</td>
<td>XP_002098061</td>
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<td>Mug66?</td>
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<td>NP_573326</td>
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<td>dTor</td>
<td>mTor</td>
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\(^{a}\) *S. pombe* NP_593855 (Taz1-interacting factor 1) is deposited as a putative Atg11 homolog in databases. However, our analysis demonstrated greater similarities to FIP200 and not yeast Atg11.

Table 1.2 Homologs of the Atg1 protein complex subunits\(^{131}\). Copyright © 2010, Elsevier.
Figure 1.1 Schematic depiction of autophagy. Copyright © 2007 Nature Publishing Group.

(A–B) Cytosolic material is sequestered by an expanding membrane sac, the phagophore, (C) resulting in the formation of a double membrane vesicle, an autophagosome; (D) the outer membrane of the autophagosome subsequently fuses with a lysosome, exposing the inner single membrane of the autophagosome to lysosomal hydrolases; (E) the cargo-containing membrane compartment is then lysed, and the contents are degraded.
Figure 1.2 Atg proteins required for various types of autophagy.

Autophagy-related (Atg) proteins that are commonly required for the autophagy-related pathways are classified as the core machinery for membrane formation. Several groups of Atg proteins are additionally required to specify the mode of autophagy; so-called autophagy, the Cvt pathway, pexophagy, and mitophagy. Atg11 is a common factor required for various types of selective autophagy.
Figure 1.3 Signaling regulation of autophagy in yeast.
The class III PtdIns3K complex mediates nucleation of the phagophore membrane, enwrapping cytosolic proteins, protein aggregates, and organelles (such as mitochondria). Atg12–Atg5–Atg16 and Atg8–PE conjugates are recruited to the phagophore, together with the transmembrane protein Atg9, facilitating the phagophore expansion step. Upon vesicle completion, most of the Atg proteins are dissociated from the autophagosome, allowing autophagosome-lysosome fusion and cargo degradation by lysosomal proteases.
Figure 1.5 Two ubiquitin-like conjugation systems. Copyright © 2009 Nature Publishing Group.

(A) The conjugation system of autophagy-related 8 (Atg8). First, Atg4 cleaves the carboxy-terminal Arg residue of Atg8 to expose Gly at the new C-terminus. Atg8 is then activated by Atg7 (an E1 enzyme), transferred to Atg3 (an E2 enzyme) and eventually conjugated to phosphatidylethanolamine (PE). The active site Cys residues of Atg7 and Atg3 are indicated. Atg4 also cleaves the amide bond between Atg8 and PE to release the protein from membranes. (B) The conjugation system of Atg12. Atg12 is conjugated to the specific Lys residue of Atg5 in a similar manner to the conjugation reaction of Atg8, except that Atg10 functions as the E2 enzyme in this system instead of Atg3. An E3 enzyme for the Atg12 conjugation reaction has not been reported. The Atg12–Atg5 conjugate interacts with Atg16 and forms an oligomer. The Atg12–Atg5–Atg16 complex then exerts an E3 enzyme-like function on the Atg8 conjugation reaction; the transfer reaction of Atg8 from Atg3 to PE is stimulated by this complex. (C) Localization of ubiquitin-like protein conjugates on autophagy-related membranes. Atg8–PE is present on both surfaces of the isolation membrane, and part of the conjugate is left inside the autophagosome, delivered to the vacuole and degraded. Atg12–Atg5–Atg16 preferentially localizes on the outer surface of the membrane and dissociates from the membrane upon completion of the autophagosome. R, Arg; G, Gly; C, Cys; K, Lys.
Figure 1.5 Two ubiquitin-like conjugation systems. Copyright © 2009 Nature Publishing Group.
Chapter 2

Autophosphorylation within the Atg1 activation loop is required for both kinase activity and the induction of autophagy in *Saccharomyces cerevisiae*
2.1 Introduction

Macroautophagy (hereafter referred to as autophagy) is a highly conserved process of self-degradation that is essential for cell survival during periods of nutrient limitation \(^{132}\). During autophagy, a double membrane grows out from a specific nucleation site, known as the preautophagosomal structure, or PAS, in \textit{Saccharomyces cerevisiae} and the phagophore assembly site in mammals \(^{44}\). This membrane encapsulates bulk protein and other constituents of the cytoplasm and ultimately targets this material to the vacuole/lysosome for degradation \(^{5}\). Recent studies have linked this pathway to a number of processes important for human health, including tumor suppression, innate immunity, and neurological disorders, like Huntington’s disease \(^{19,133}\). Determining how this pathway is regulated is therefore important for our understanding of these processes and our attempts to manipulate autophagy in clinically beneficial ways.

Most of the molecular components of the autophagy pathway were initially characterized in the budding yeast, \textit{S. cerevisiae}, but orthologs of many of these Atg proteins have since been found in other eukaryotes \(^{33,132}\). A complex of proteins that contains the Atg1 protein kinase is of special interest and appears to be a key point of regulatory control within this pathway \(^{35,48,73,74}\). In \textit{S. cerevisiae}, genetic and biochemical data indicate that this complex is targeted by at least three different signaling pathways. Two of these pathways, involving the Tor and cAMP-dependent protein kinases, inhibit this process, whereas the AMP-activated protein kinase is needed for the full induction of autophagy \(^{72,92,127,134}\). The manner in which these signaling pathways regulate Atg1 activity and the precise role of this kinase in the autophagy process are presently matters of intense scrutiny.
Although Atg1 kinase activity is required for the induction of autophagy, relatively little is known about how this enzyme is regulated \textit{in vivo}. Two proteins associated with Atg1, Atg13 and Atg17, have been shown to be required for full Atg1 kinase activity both \textit{in vitro} and \textit{in vivo} \cite{35,73}. The roles of these proteins appear to be conserved through evolution as functional homologs of both have been identified in fruit flies and/or mammals \cite{46,97-102}. However, it is not yet clear precisely how these proteins stimulate Atg1 activity. In this study, we show that Atg1 is autophosphorylated within the activation loop and that this phosphorylation is required for both Atg1 kinase activity and the induction of autophagy. The activation loop is a structurally conserved element within the kinase domain and phosphorylation within this loop is often a necessary pre-requisite for efficient substrate binding and/or phosphotransfer in the catalytic site \cite{135,136}. This loop generally corresponds to the sequence between two signature elements within the core kinase domain, the DFG and APE motifs \cite{137}. Phosphorylation within this loop tends to result in a more ordered structure for this region and the proper positioning of key elements within the catalytic core of the kinase domain \cite{136,138,139}. We found that Atg1 activation loop phosphorylation was correlated with the onset of autophagy and that replacing the site of phosphorylation with a phosphomimetic residue led to constitutive Atg1 autophosphorylation \textit{in vivo}. In all, the data here suggest that Atg1 phosphorylation within its activation loop may be an important point of regulation within the autophagy pathway and models that discuss these data are presented.
2.2 Materials and methods

2.2.1 Strains and growth media

Standard *Escherichia coli* growth conditions and media were used throughout this study. The yeast rich-growth medium, YPAD, consists of 1% yeast extract, 2% Bacto-peptone, 500 mg/L adenine–HCl, and 2% glucose. The yeast YM glucose and SC glucose minimal growth media have been described \(^{140,141}\). The nitrogen starvation medium, SD-N, consists of 0.17% yeast nitrogen base lacking amino acids and ammonium sulfate, and 2% glucose. Growth media reagents were from Difco. Standard yeast genetic methods were used in this study. The yeast strains used were TN125 (*MATa* ade2 his3 leu2 lys2 trp1 ura3 pho8::pho8Δ60), YYK126 (TN125 *atg1Δ::LEU2*), YYK130 (TN125 *atg13Δ::TTRP1*), PHY3687 (TN125 *atg1Δ::LEU2 atg13Δ::kanMX*), BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), PHY4167 (BY4741 *atg1Δ::kanMX*), PHY4177 (BY4741 *atg13Δ::kanMX*), and PHY4181 (BY4741 *atg17Δ::kanMX*). \(^{35,142,143}\).

2.2.2 Plasmid constructions

Site-directed mutageneses were performed with the Clontech Transformer kit on the ATG1-containing plasmids, pPHY1115 and pPHY2376, to generate the variants (K54A, D211A, T226A and T226E) used herein. Plasmid pPHY1115 was originally named pRS316-3xmycATG1 and was generously provided by Dr. Yoshinori Ohsumi. Plasmid pPHY2376 is a pRS423-based construct in which Atg1 has three copies of the HA epitope at its N terminus; this plasmid was originally provided by Dr. Daniel Klionsky. Variants in the YFP–Atg1 fusion background were generated similarly with the wild-type plasmid, pPHY2297, as the starting vehicle \(^{74}\). For the rAtg1 variants, fragments...
encoding residues 1–420 were PCR amplified from the appropriate yeast plasmids and cloned into the pET21a expression vector. The plasmid, pPHY2427, encoded an HA epitope-tagged version of Atg13 under the control of the promoter from the copper-inducible *CUP1* gene. The ATG13 locus was cloned from the plasmid, pRS316–ATG13, that was kindly provided by Dr. Takeshi Noda. A site-directed mutagenesis was performed to insert an *Xma*I site immediately following the start codon of ATG13. The ATG13 coding sequences and transcriptional terminator were then cloned as a 3.1 kb *Xma*I–*Not*I fragment into pPHY2203 to form pPHY2427. The pPHY2203 plasmid was described previously and contains the *CUP1* promoter and the HA epitope.

### 2.2.3 Western blotting and immunoprecipitation

Protein samples for Western blotting were prepared as described. The proteins were separated on SDS–polyacrylamide gels, transferred to nitrocellulose membranes and the membranes were probed with the appropriate primary and secondary antibodies. The Supersignal chemiluminescent substrate (Pierce Chemical, Rockford, IL) or Odyssey infrared imaging system (LI-COR Biosciences) was subsequently used to detect the reactive bands or fluorescence intensities. Immunoprecipitations were performed as described. HA-tagged proteins were generally precipitated on anti-HA matrix beads (Roche) whereas myc-tagged proteins were immunoprecipitated with a monoclonal anti-myc antibody and then collected on Protein A–Sepharose (GE Healthcare) as described. The amount of immunoprecipitated and co-immunoprecipitated proteins were subsequently assessed by Western immunoblotting with the appropriate antibodies. To assess phosphorylation of position T226 within Atg1, a polyclonal antiserum was
generated in rabbits against a phosphorylated peptide, FLPNTSLAE(pT)LCGSPLY, that corresponds to the sequence of the Atg1 activation loop (see Figure 2.1, A). The peptide synthesis, antibody generation and purification were performed by Lampire Biological Laboratories. Western blots with this antibody were generally performed with cells overexpressing Atg1 from a 2 μ plasmid.

2.2.4 In vitro kinase assays (IVKAs)

The immunoprecipitated Atg1 proteins, or purified recombinant Atg1 fragments, were incubated for 30 – 60 min at 30 °C with 10 mCi [γ-32P]ATP in a 40 μl reaction (50 mM potassium phosphate, 5 mM NaF, 10 mM MgCl 2, 4.5 mM DTT, protease inhibitors, and phosphatase inhibitors) with or without 10 μg of myelin basic protein (MBP) (Sigma, St. Louis). The reactions products were separated on SDS–polyacrylamide gels and the gels were fixed, dried, and analyzed either by autoradiography or by phosphorimaging with a Typhoon Trio (GE Healthcare).

2.2.5 Recombinant protein purification

*E. coli* cells were grown to an OD 600 of ~0.5/ml and IPTG was added to a final concentration of 1 mM to induce expression of the rAtg1 proteins. The induction was carried out for 4 hr. The cells were collected by centrifugation and lysed by sonication in lysis buffer (50 mM sodium phosphate, 500 mM NaCl, and protease inhibitors). Clarified cell lysates were then incubated with 1 ml Ni–NTA agarose beads (Qiagen) at 4 °C overnight. The beads were collected by centrifugation and washed three times with PBS.
containing 20 mM imidazole, and the recombinant proteins were eluted with PBS containing 250 mM imidazole.

2.2.6 Autophagy assays

Autophagy activity was assessed with several different assays that have been described previously. In general, autophagy was induced by treating mid-log-phase cells with 200 ng/ml rapamycin for the indicated times. The ALP-based assay measures the delivery and subsequent activation in the vacuole of an altered form of the Pho8 alkaline phosphatase, known as Pho8Δ60\textsuperscript{142,145}. The relative level of autophagy was indicated by the difference in alkaline phosphatase activity detected between extracts prepared from log-phase and rapamycin-treated cells. The data presented represent the average of at least three independent experiments where the autophagy levels generally varied by less than 15% between the different experimental replicates. In the GFP–Atg8 assay, the relative level of free GFP generated by proteolysis in the vacuole is used as an indicator of autophagy activity\textsuperscript{56}. The GFP–Atg8 fusion protein and the free GFP were detected by Western blotting with an anti-GFP antibody. The final assay assesses the processing of overexpressed aminopeptidase I, or Ape1, that occurs via the autophagy pathway\textsuperscript{74}. In this assay, the cells express Ape1 at a level that saturated the cytoplasm-to-vacuole, or Cvt, transport system. Previous work has shown that the precursor Ape1 protein in these cells is processed by the autophagy pathway following either rapamycin treatment or nitrogen starvation\textsuperscript{79}. The Ape1 proteins were detected by Western blotting with an anti-Ape1 antibody provided by Dr. Daniel Klionsky.
2.2.7 Fluorescence microscopy

The CFP–Atg11 and YFP–Atg1 fusions were under the control of the inducible promoter from the yeast CUP1 gene. Expression of the fusion proteins was induced by the addition of 100 μM CuSO₄ for 2 hr at 30 °C, and the samples were imaged as described 74.

2.3 Results

2.3.1 Residue T226 within the activation loop was a site of Atg1 autophosphorylation

The activation loop of the S. cerevisiae Atg1 contains a candidate site of phosphorylation at threonine-226 (T226). This position, and much of the surrounding sequence, is conserved in Atg1 orthologs (Figure 2.1, A). To test whether T226 was indeed a site of phosphorylation, we generated an antibody that specifically recognized the phosphorylated form of a peptide whose sequence corresponded to that of the Atg1 activation loop (Figure 2.1, A; see materials and methods). This antibody recognized the wild-type Atg1 protein but not the Atg1T226A and Atg1T226E variants that had substitutions at position T226 (Figure 2.1, B). Interestingly, the signal with this antibody was found to increase upon exposure to conditions that lead to an induction of autophagy, such as rapamycin treatment (Figure 2.1, B; see below). The recognition by this antibody was lost upon phosphatase treatment of the immunoprecipitated Atg1 and was absent from cells expressing only the kinase-defective variants, Atg1K54A or Atg1D211A (Figure 2.1, C–D). The positions altered in these variants, K54 and D211, are highly conserved in protein kinases and are important for the proper positioning of ATP within the active site 146-148. These results suggested that the recognition by this antibody required the presence of a
phosphate group at position T226 and that the addition of this phosphate might be
catalyzed by Atg1 itself. To examine this latter possibility further, we attempted to
regenerate the phosphorylation signal at T226 in vitro with immunoprecipitated Atg1
proteins that had been pretreated with λ phosphatase. We found that the T226
phosphorylation was restored with the wild-type Atg1 but not with kinase-defective
variants (Figure 2.1, E; data not shown). Altogether, these data suggested that position
T226 within the activation loop was a site of Atg1 autophosphorylation and that the
presence of this modification was coincident with the induction of autophagy.

2.3.2 Phosphorylation at position T226 within the activation loop was required for
Atg1 protein kinase activity

To assess the importance of activation loop phosphorylation for Atg1 function, we
replaced T226 with a nonphosphorylatable alanine residue and assessed Atg1 kinase
activity both in vivo and in vitro. The in vitro assays examined Atg1 autophosphorylation
and the phosphorylation of a nonphysiological substrate, the MBP. In each case, the
Atg1 proteins examined were precipitated from rapamycin-treated cells. We found that
this Atg1T226A variant exhibited significantly diminished activity, relative to the wild-type
enzyme, in both of these assays (Figure 2.2, A–D). Two kinase-defective versions of
Atg1, Atg1K54A and Atg1D211A, were used as negative controls in these experiments and
the level of kinase activity associated with Atg1T226A was similar to that observed with
these control proteins. We also found that a preincubation of the precipitated Atg1 with
ATP resulted in elevated kinase activity toward MBP (Figure 2.2, C). This stimulation
required the presence of the kinase domain and suggested that Atg1 autophosphorylation
might increase the specific activity of this enzyme (Figure 2.11, A–B). This autophosphorylation could occur at position T226 and/or other positions within the Atg1 protein.

To assess Atg1 kinase activity in vivo, we took advantage of previous observations indicating that Atg1 autophosphorylation results in the production of a slower migrating form of this protein on SDS-polyacrylamide gels (Atg1-P in Figure 2.3, A) \(^{35,73,74}\). Our data indicate that the site of autophosphorylation responsible for this shift is distinct from T226 (see below and our unpublished observations). The relative level of this second, upper band can serve as a measure of the Atg1 kinase activity present in cells \(^{73}\). Consistent with the in vitro data above, we found significantly less of the Atg1\(^{T226A}\) variant, relative to the wild type, in the slower-migrating form following either rapamycin treatment or nitrogen starvation, two conditions that are known to induce an autophagy response (Figure 2.3, A–B). The relative activity of the Atg1\(^{T226A}\) variant again resembled the kinase-defective controls, Atg1\(^{K54A}\) and Atg1\(^{D211A}\). Interestingly, we found that the \(\alpha\)-T226P antibody specifically recognized this slower-migrating form of Atg1 on Western blots (Figure 2.3, B–C). In all, these data suggested that T226 phosphorylation within the activation loop was required for Atg1 kinase activity and was associated with an autophosphorylated form of Atg1 found specifically in cells undergoing autophagy.

### 2.3.3 Activation loop phosphorylation was required for the induction of autophagy

We used three different assays to test whether phosphorylation within the Atg1 activation loop was required for the induction of autophagy. The first assessed the vacuolar delivery and processing of an altered form of the Pho8 alkaline phosphatase, known as Pho8Δ60
This truncated protein lacks a membrane-spanning domain and the targeting information necessary for normal delivery through the secretory pathway \(^{142,149}\). As a result, this protein can be delivered to the vacuole, and therein activated, only by the autophagy pathway. Using this assay, we found that no significant autophagy activity was detectable in cells carrying only the \(\text{Atg}1^{\text{T226A}}\) variant (Figure 2.4, A). The levels of autophagy detected were similar to those associated with the \(\text{atg}1\Delta\) control strain.

The second assay measured the processing of a GFP–Atg8 fusion protein \(^{56}\). Atg8 is normally incorporated into the autophagosome and delivered into the vacuolar lumen \(^{43,44,124}\). The Atg8 is then degraded by vacuolar hydrolases, thereby releasing the more stable GFP protein \(^{56}\). The relative level of free GFP can be used as a measure of the autophagy activity present. As with the ALP-based assay, no significant processing of the GFP–Atg8 fusion was detected in cells with the \(\text{Atg}1^{\text{T226A}}\) variant (Figure 2.4, B). This autophagy defect was corroborated by an examination of the subcellular localization of the GFP fluorescence in these cells. In wild-type cells, the GFP–Atg8 fusion is delivered to the vacuole and the free GFP accumulates within the lumen of this organelle (Figure 5). In contrast, very little, if any, GFP fluorescence is detected in the vacuoles of mutants defective for autophagy (see \(\text{atg}1\Delta\) in Figure 2.4) \(^{43}\). Consistent with the above processing defects, we found no significant GFP fluorescence within the vacuoles of \(\text{atg}1\Delta\) cells carrying the \(\text{Atg}1^{\text{T226A}}\) variant (Figure 2.5). It should be noted that this GFP–Atg8 fusion was normally localized to the PAS in these cells (Figure 2.5).

Finally, we assessed the autophagy-dependent maturation of the Ape1 protein. Ape1 is a normal cargo for the cytoplasm-to-vacuole, or Cvt, transport pathway that is responsible for delivering a set of proteins to the vacuole during log-phase growth \(^{32,150}\).
Overexpression of Ape1 saturates the Cvt system and results in the cytoplasmic accumulation of an unprocessed, precursor form of this protein (see Figure 2.4, C) \(^{32}\). Upon the induction of autophagy, this protein is efficiently delivered to the vacuole and processed to its mature, active form \(^{79,151}\). We found that the Atg1\(^{T226A}\) variant was defective for this autophagy-mediated processing and that the Ape1 protein was present only in its precursor form in these cells (Figure 2.4, C). Altogether, these data indicated that Atg1 autophosphorylation at T226 was necessary for the induction of the autophagy pathway in \(S.\ cerevisiae\).

\[ \text{2.3.4 The Atg1}^{T226E} \text{ variant exhibited constitutive autophosphorylation activity} \]

To test whether the presence of T226 phosphorylation was sufficient for Atg1 autophosphorylation, we replaced this position with the potential phosphomimetic residues, glutamic and aspartic acid. Similar results were obtained with both substitutions, and we focus here on the Atg1\(^{T226E}\) variant. Interestingly, we found that this Atg1\(^{T226E}\) variant was competent for autophosphorylation \textit{in vitro} when isolated from rapamycin-treated cells (Figure 2.12, A). However, unlike the wild-type protein, Atg1\(^{T226E}\) also exhibited elevated levels of autophosphorylation in log-phase cells that were typically not undergoing autophagy. This constitutive, or unregulated, autophosphorylation activity was observed in both \textit{in vitro} and \textit{in vivo} assays (Figure 2.6, A–C). For example, in an \textit{in vitro} assay, elevated levels of radioactivity, relative to the wild-type protein, were incorporated into the Atg1\(^{T226E}\) variant isolated from log-phase cells (Figure 2.6, A). In addition, the fraction of this variant found in the slower-migrating form on SDS-polyacrylamide gels was elevated in log-phase cell extracts.
(Figure 2.6, B–C). In both assays, the overall level of autophosphorylation was slightly elevated with the Atg1\textsuperscript{T226E} variant. These \textit{in vivo} results with the Atg1\textsuperscript{T226E} protein supported the above contention that the presence of the slower-migrating form of Atg1 was due to autophosphorylation at a site distinct from T226. In particular, we found that the upper band associated with the Atg1\textsuperscript{T226E} protein was lost upon phosphatase treatment and was regenerated in a subsequent incubation with ATP (Figure 2.6, D and Figure 2.12, B).

Finally, we found that a recombinant fragment of Atg1 produced in \textit{E. coli} (rAtg1) was also capable of catalyzing an autophosphorylation reaction (Figure 2.7). This fragment contained residues 1–420 of the wild-type protein and included the entire protein kinase domain located at the N terminus of Atg1. This autophosphorylation was also dependent upon the presence of T226 as replacing this position with an alanine residue resulted in significantly diminished activity (Figure 2.7). This rAtg1\textsuperscript{T226A} variant also exhibited decreased activity in assays with MBP as substrate (Figure 2.13). In contrast, a recombinant protein with glutamic acid replacing T226 was capable of significant autophosphorylation (Figure 2.7). This result was similar to that obtained above with the wild-type protein and together these observations suggested that it was the phosphorylation at position T226 that was important for this Atg1 autophosphorylation. Moreover, the presence of a phosphomimetic at position 226 appeared to disrupt the normal control of Atg1 and resulted in constitutive autophosphorylation.
2.3.5 The Atg1<sup>T226E</sup> variant was nonfunctional for autophagy

We also tested whether the Atg1<sup>T226E</sup> and Atg1<sup>T226D</sup> variants were functional for autophagy. Using the three processing assays described above, we found little, if any, autophagy activity in atg1Δ cells carrying these variants (Figure 2.8, A–C). Moreover, we found that the GFP–Atg8 fusion protein was not transported efficiently into the lumen of the vacuole in atg1–T226E cells (Figure 2.8D).

This latter defect is consistent with a failure to carry out the normal autophagy process. Therefore, although these variants exhibited constitutive autophosphorylation activity, neither was capable of replacing the wild-type protein in the autophagy process. These variants also failed to induce autophagy in log-phase cells and had little detrimental effect upon autophagy when overexpressed in wild-type cells (Figure 2.8, A and Figure 2.14). The autophagy defects did not appear to be due simply to gross misfolding as the Atg1<sup>T226E</sup> protein was present at normal steady-state levels in yeast cells, was localized normally to the PAS, and interacted with both Atg13 and Atg17, two key regulators of Atg1 kinase activity (Figure 2.9, A–C) 35,51. However, we did find that the Atg1<sup>T226E</sup> variant was less effective than the wild-type protein at phosphorylating the nonphysiological substrate, MBP, in an in vitro assay (Figure 2.9, D). Therefore, it is possible that the autophagy defects observed with this variant might be due to a failure to phosphorylate an important, but as yet unknown, substrate in vivo.

2.3.6 Atg13 and Atg17 were required for T226 phosphorylation

The precise roles of Atg13 and Atg17 in the regulation of Atg1 kinase activity have not yet been defined. Here, we tested whether these regulators were required for the
phosphorylation of Atg1 within its activation loop. Indeed, we found that T226 phosphorylation was greatly diminished in both \textit{atg13Δ} and \textit{atg17Δ} cells (Figure 2.10, A). The residue levels remaining in these mutants were higher than those observed in strains carrying only a kinase-defective version of Atg1 (Figure 2.15). This low level of T226 phosphorylation might be important for the suppression of \textit{atg13} defects observed upon the overexpression of Atg1 in these mutants \cite{78}. We also found that the overexpression of Atg13 resulted in an elevated level of T226 phosphorylation in both log-phase and rapamycin-treated cells (Figure 2.10, B). Consistent with this observation, the overexpression of Atg13 also led to slightly increased levels of autophagy in rapamycin-treated cultures (Figure 2.10, C). Finally, we tested whether the autophosphorylation associated with the Atg1\textsuperscript{T226E} variant required either Atg13 or Atg17. We found that the slower-migrating form of Atg1\textsuperscript{T226E} that was indicative of Atg1 kinase activity \textit{in vivo} was still present in both \textit{atg13Δ} and \textit{atg17Δ} cells (Figure 2.10, D). Therefore, the presence of a phosphomimetic within the Atg1 activation loop bypassed the normal requirement for these regulators for Atg1 autophosphorylation.

\textbf{2.4 Discussion}

The autophagy pathway has been implicated in a wide variety of phenomena important for human health and has been suggested as a potential therapeutic target for a number of disease conditions \cite{19,133,152}. To manipulate autophagy in a productive way, it is critical that we develop a thorough understanding of how this pathway is regulated \textit{in vivo}. Toward this end, the work here identifies a specific phosphorylation event within the activation loop of Atg1 as a potential point of control for autophagy in \textit{S. cerevisiae}. 

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Several observations point to the importance of this phosphorylation at position T226 for the autophagy process. First, the substitution of a nonphosphorylatable alanine residue for the threonine normally present resulted in a loss of Atg1 kinase activity and a failure to induce the autophagy process. Second, a phosphospecific antibody, prepared against a phosphopeptide corresponding to the Atg1 activation loop, recognized the wild-type Atg1 but not the Atg1T226A variant. Third, the relative level of T226 phosphorylation was found to increase specifically upon exposure to conditions that induce the autophagy process. Fourth, this antibody specifically recognized an autophosphorylated form of Atg1 that was present in cells undergoing autophagy. Fifth, we found that replacing the T226 position with an acidic residue, either glutamic or aspartic acid, resulted in an enzyme that exhibited constitutive activity with respect to autophosphorylation. This latter observation is important as it indicates that the presence of a potential phosphomimetic at position 226 in the activation loop results in the production of an active enzyme. Taken altogether, these data support the contention that Atg1 is phosphorylated within its activation loop and that this phosphorylation is important for the normal induction of the autophagy process. Since this site has been evolutionarily conserved in Atg1 orthologs, it is possible that this phosphorylation will be a point of control for autophagy in other eukaryotes.

Many protein kinases require phosphorylation within their activation loop for full activity and this modification can be a critical event in the activation of these enzymes. This phosphorylation can be catalyzed by a second enzyme or can be the consequence of an autophosphorylation reaction. For Atg1, the data here suggest that this modification is self-catalyzed. A functional Atg1 kinase domain was required for T226
phosphorylation in vivo and this modification occurred in vitro with Atg1 immunoprecipitates. This phosphorylation was also shown to require the presence of Atg13 and Atg17, two previously identified regulators of Atg1 kinase activity. Interestingly, replacing position T226 with a phosphomimetic, such as glutamic acid, resulted in an enzyme that no longer required Atg13 or Atg17 for autophosphorylation in vivo. This bypass of the requirement for these regulators may suggest that facilitating Atg1 autophosphorylation within the activation loop is one of the functions of the Atg13 and Atg17 proteins during the autophagy process.

Although the data here suggest that T226 phosphorylation is required for the induction of autophagy neither the Atg1\textsuperscript{T226E} nor the Atg1\textsuperscript{T226D} variant was capable of supporting this degradative process in vivo. The underlying basis for this defect is not yet known, but there are a number of potential explanations that can be considered. On one extreme is the possibility that it is the threonine itself at position 226, and not its phosphorylation, that is important for Atg1 function. We do not favor this possibility given the strong correlation between T226 phosphorylation and both the induction of autophagy and the presence of Atg1 kinase activity, as measured by autophosphorylation. In addition, this model is difficult to reconcile with the observation that the presence of a phosphomimetic at position 226 led to constitutive Atg1 autophosphorylation. Therefore, we feel that the results obtained here with the Atg1\textsuperscript{T226E} and Atg1\textsuperscript{T226D} variants require a different explanation. One possibility is that the phosphomimetic does not fully restore the functionality of a phosphorylated threonine and therefore results in an enzyme with only partial activity. This could explain why the Atg1\textsuperscript{T226E} protein is competent for autophosphorylation but not the phosphorylation of an exogenous substrate, like MBP.
Alternatively, this failure to phosphorylate MBP might reflect some inherent aspect of Atg1 biology. For example, Atg1 might normally participate in a negative feedback loop that is permanently engaged in the Atg1\textsuperscript{T226E} variant. Another possibility is that the autophagy defects might be due to the inability of these variants to exist in a kinase-inactive form. Previous work has indicated that Atg1 has both kinase-dependent and kinase-independent activities in the autophagy process\textsuperscript{50,52,157}. Atg1 may therefore need to oscillate between kinase-active and kinase-inactive states to stimulate autophagy. Clearly, further work will be necessary to distinguish between these and other potential explanations. However, we feel that the work here in its totality identifies the autophosphorylation of Atg1 within its activation loop as an important event in the normal induction of autophagy. Since Atg1 and its regulators have been conserved through evolution, a continued analysis of this modification is likely to provide additional insights into the general control of this degradative process.
Figure 2.1 T226 within the activation loop was a site of Atg1 autophosphorylation.

(A) The activation loop sequences for the Atg1 orthologs from the indicated organisms are shown. Residues identical in all the orthologs are shown on a black background and those residues that are chemically similar are shown on a gray background. The T226 residue is indicated by an asterisk and the bar at the top indicates the peptide used to generate the phosphospecific antibody. Hs, Homo sapiens. (B) Phosphorylation at position T226 within the Atg1 activation loop increased upon rapamycin treatment. Note that the Atg1T226A and Atg1T226E variants were not recognized by the anti-T226P phosphospecific antibody. In B–E, the indicated Atg1 proteins were immunoprecipitated from cell extracts with an antibody directed against the indicated epitope, separated on SDS-polyacrylamide gels, and then analyzed by Western blotting with the indicated antibodies. The cell extracts were prepared from either log-phase (L) or rapamycin-treated (R) cultures. The protein signals were assessed by autoradiography following chemiluminescence illumination. (C and D) Atg1 recognition by the T226-P phosphospecific antibody was lost upon phosphatase treatment and was absent from cells that contained kinase-defective variants of Atg1. Where indicated, the precipitated Atg1 proteins were treated with λ phosphatase (PPase) prior to SDS-polyacrylamide gel electrophoresis. K54A and D211A refer to kinase-defective variants of Atg1. (E) The T226 phosphorylation signal was regenerated in an in vitro kinase assay. The indicated Atg1 proteins were precipitated from yeast cells that had been treated with rapamycin, and the relative level of T226 phosphorylation was assessed by Western blotting with the phosphospecific antibody. Where indicated, the precipitated Atg1 proteins were treated with λ phosphatase (PPase) and then subjected to an in vitro kinase autophosphorylation assay (IVKA).
Figure 2.1 T226 within the activation loop was a site of Atg1 autophosphorylation.
Figure 2.2 The presence of T226 within the Atg1 activation loop was required for protein kinase activity.

Wt, wildtype. (A) Replacing T226 with a nonphosphorylatable residue resulted in a loss of Atg1 autophosphorylation. Atg1 proteins with the indicated alterations were immunoprecipitated from rapamycin-treated cells and subjected to an *in vitro* autophosphorylation assay as described in the materials and methods. The Atg1<sup>K54A</sup> and Atg1<sup>D211A</sup> variants were kinase-defective controls. In both A and B, the Atg1 proteins were tagged at the N terminus with three copies of the myc-epitope. (B) Quantification of the phosphorimager data shown in A. (C) An *in vitro* kinase assay, using the MBP as substrate, was performed with the indicated Atg1 proteins isolated from rapamycin-treated cells. In the two right-hand lanes, the wild-type Atg1 protein was immunoprecipitated and either mock treated (−) or pre-incubated with 3 mM ATP for 60 min at 30 °C (+). The samples were subsequently washed to remove the excess ATP and an *in vitro* kinase assay was performed with [γ-<sup>32</sup>P]ATP and MBP. (D) Quantification of the phosphorimager analysis data in C.
Figure 2.3 The Atg1$^{T226A}$ variant was defective for Atg1 autophosphorylation in vivo.

(A) The presence of the T226A alteration resulted in a loss of Atg1 autophosphorylation in vivo. Western blots were performed with extracts prepared from either log-phase (L) or rapamycin-treated (R) cells with the indicated Atg1 proteins. In the bottom part, immunoprecipitated wild-type Atg1 was either mock treated (IVKA$^{-}$) or subjected to an in vitro autophosphorylation reaction with 3 mM ATP (IVKA$^{+}$). An aliquot of this latter sample was then split into two and either mock treated (PPase$^{-}$) or incubated with $\lambda$ phosphatase (PPase$^{+}$). PPase, $\lambda$ phosphatase; IVKA, in vitro kinase assay; Atg1-P, autophosphorylated form of Atg1 that exhibits an altered mobility on SDS-polyacrylamide gels. (B) Atg1 activation loop phosphorylation was elevated in response to nitrogen starvation and required the presence of a functional Atg1 kinase domain. In B and C, the indicated Atg1 proteins were immunoprecipitated from cell extracts with an antibody directed against the indicated epitope, separated on SDS-polyacrylamide gels, and then analyzed by Western blotting with the indicated antibodies. The cell extracts were prepared from either log-phase (L), rapamycin-treated (R) or nitrogen starved (N) cultures. The protein signals were detected with a LI-COR Odessey infrared detection system. For nitrogen starvation, log-phase cells were transferred to SD-N medium for 6 hr. D211A, a kinase-defective variant of Atg1. (C) The anti-T226P phosphospecific antibody ($\alpha$-T226P) recognized a slower-migrating, autophosphorylated form of Atg1 that appeared upon rapamycin treatment. The protein signals were detected with a LI-COR Odessey infrared detection system and the right-most part shows a merged image for the $\alpha$-myc and $\alpha$-T226P signals.
Figure 2.3 The Atg1\textsuperscript{T226A} variant was defective for Atg1 autophosphorylation \textit{in vivo}. 
Figure 2.4 The Atg1^{T226A} variant was defective for autophagy.

(A) Autophagy activity was assessed with the ALP Pho8Δ60-based assay using an atg1Δ strain expressing the indicated Atg1 proteins. The atg1Δ control strain contained a vector plasmid. Extracts were prepared from cells that had been treated with 200 ng/ml rapamycin for either 0 (Log) or 4hr (Rap). The data shown are the average of two independent experiments where the values obtained varied by less than 15% between replicates. (B) Autophagy activity was assessed with a GFP–Atg8 processing assay as described in MATERIALS AND METHODS. The level of free GFP was an indicator of the relative autophagy activity present. (C) Autophagy activity was assessed with an assay measuring the vacuolar processing of overexpressed Ape1 as described in MATERIALS AND METHODS. The presence of the mature, processed Ape1 protein (mApe1) was an indicator of autophagy activity. preApe1, precursor Ape1. The bottom part shows the relative levels of the different myc-tagged Atg1 proteins examined. For B and C, cells were treated with 200 ng/ml rapamycin for the hours indicated.
Figure 2.4 The Atg1$^{T226A}$ variant was defective for autophagy.
Figure 2.5 A GFP-Atg8 fusion protein was not delivered to the vacuole in cells containing the Atg1\textsuperscript{T226A} variant.

Yeast strains carrying the indicated Atg1 proteins were treated for 2 hr with 200 ng/ml rapamycin and the subcellular localization of an GFP–Atg8 fusion protein was then assessed by fluorescence microscopy. Atg8 is normally incorporated into the autophagosome and transported to the vacuolar compartment. This transport results in the presence of GFP fluorescence in the vacuoles of cells with a functional autophagy pathway. The vacuoles of the representative cells shown in the bottom row are indicated by the white arrows. Note that the GFP–Atg8 fusion was still found at the PAS in cells containing the Atg1\textsuperscript{T226A} variant.
Figure 2.6 The Atg1\textsuperscript{T226E} variant was a constitutively active protein kinase.

(A) The Atg1\textsuperscript{T226E} variant exhibited elevated protein kinase activity in log-phase cell extracts. Atg1 was immunoprecipitated from either log-phase (Log) or rapamycin-treated (Rap) cells and subjected to an \textit{in vitro} autophosphorylation assay with [\gamma-\textsuperscript{32}P]ATP. T, wild-type Atg1; E, Atg1\textsuperscript{T226E} variant. The top is the autoradiograph image and the bottom is the Western blot control performed with an anti-myc antibody. For A–D, the Atg1 proteins were tagged with three copies of the myc epitope. (B) Atg1\textsuperscript{T226E} exhibited constitutive autophosphorylation \textit{in vivo}. Western blots were performed with extracts prepared from either log-phase (L) or rapamycin-treated (R) cultures of an \textit{atg1Δ} strain with the indicated Atg1 proteins. (C) The relative level of the slower-migrating form of the Atg1\textsuperscript{T226E} variant was elevated in log-phase cells. Western blotting was performed with log-phase extracts prepared from \textit{atg1Δ} cells carrying the indicated Atg1 proteins. (D) The slower-migrating form of Atg1 was the result of an autophosphorylation at a position distinct from T226. The wild-type Atg1 and Atg1\textsuperscript{T226E} proteins were immunoprecipitated from rapamycin-treated cells and incubated with 1 phosphatase. The sample was then split into two aliquots and either mock treated with the reaction buffer or incubated with 3 mM ATP. PPase, \textgreek{p} phosphatase; IVKA, \textit{in vitro} kinase assay.
Figure 2.7 Autophosphorylation of a recombinant Atg1 protein required the presence of position T226, the site of activation loop phosphorylation.

(A) Recombinant fragments of Atg1 containing residues 1–420 were partially purified from E. coli cell extracts and incubated with [γ-^32^P]ATP to assess autophosphorylation in vitro. The relative level of label incorporation was then assessed with a phosphorimager and the image from this analysis is shown at the top. The bottom shows the Western blot control performed with an antibody specific to the His6-tag present on these recombinant proteins. (B) Quantification of the phosphorimager analysis shown in A.
Figure 2.8 The Atg1T226E variant was defective for autophagy.

(A) The ALP-based assay was used to assess autophagy activity in an atg1Δ strain expressing the indicated Atg1 proteins. Extracts were prepared from cells that had been treated with 200 ng/ml rapamycin for either 0 (Log) or 4 hr (Rap). The data shown are the average of two independent experiments where the values obtained varied by less than 15% between replicates. The atg1Δ control strain contained a vector plasmid. (B and C) Autophagy activity was assessed with either the GFP–Atg8 (B) or the Ape1 (C) processing assays as described in MATERIALS AND METHODS. In each case, the strains were treated with 200 ng/ml rapamycin for the indicated time (hours). B, bottom, shows the relative levels of the different Atg1 proteins examined. (D) A GFP–Atg8 fusion protein was not delivered to the vacuole in cells expressing only Atg1T226E. The subcellular localization of a GFP–Atg8 fusion protein was assessed by fluorescence microscopy in atg1Δ cells carrying the indicated Atg1 proteins. The cells were treated with 200 ng/ml rapamycin for 2 hr prior to examination. Note that the GFP–Atg8 fusion was localized to the PAS in cells containing the Atg1T226E variant.
Figure 2.8 The Atg1^{T226E} variant was defective for autophagy.
Figure 2.9 The Atg1^{T226E} variant was localized to the PAS and interacted with Atg13 and Atg17.

(A) The Atg1^{T226A} and Atg1^{T226E} proteins were localized normally to the PAS. The subcellular localization of YFP fusions to the indicated Atg1 proteins was assessed in an *atg1Δ* strain by fluorescence microscopy. An Atg11–CFP fusion protein was used as a marker for the PAS. The cells were all treated with 200 ng/ml rapamycin for 2 hr before examination. The white arrows indicate the location of the PAS in the respective cells. (B) The Atg1^{T226E} variant interacted with Atg17. The indicated HA-tagged Atg1 proteins were precipitated from cell extracts and the relative amount of associated Atg17 protein was assessed by Western blotting with an anti-myc antibody. (C) The Atg1^{T226E} variant interacted with Atg13. HA-tagged Atg13 was precipitated from cell extracts and the relative amounts of the associated Atg1 proteins were assessed by Western blotting with an anti-myc antibody. +13, cells containing the HA-tagged Atg13 protein; +Vec, cells containing a vector control and thus lacking the tagged Atg13 protein. (D) An *in vitro* kinase assay using MBP as substrate was performed with the wild-type Atg1 and the Atg1^{T226E} variant. The indicated Atg1 proteins were immunoprecipitated and incubated with [γ-^{32}P]ATP and the nonphysiological substrate, MBP; each sample was performed in duplicate. The bottom parts show the amount of Atg1 protein present in the *in vitro* reactions.
Figure 2.9 The Atg1^{T226E} variant was localized to the PAS and interacted with Atg13 and Atg17.
Figure 2.10 Atg13 and Atg17 were required for Atg1 autophosphorylation within the activation loop.

(A) T226 phosphorylation in \textit{atg13}\textsuperscript{Δ} and \textit{atg17}\textsuperscript{Δ} mutants. The wild-type Atg1 was precipitated from \textit{atg13}\textsuperscript{Δ}, \textit{atg17}\textsuperscript{Δ}, or wild-type cells and analyzed by Western blotting with the anti-T226P phosphospecific antibody. The Atg1\textsuperscript{K54A} variant (K54A) was used as a kinase-defective control. L, log phase; R, rapamycin-treated. (B) Overexpression of Atg13 resulted in an increased level of T226 phosphorylation. The relative level of T226 phosphorylation was assessed in both log-phase and rapamycin-treated wild-type cells carrying either a vector control (−) or an \textit{ATG13} overexpression plasmid. The wild-type Atg1 was immunoprecipitated from these cells with an antibody specific for the HA epitope present on this protein. (C) Overexpression of Atg13 resulted in elevated levels of autophagy. A plasmid overexpressing Atg13 or a vector control were introduced into wild-type cells, and autophagy levels were assessed with the Pho8Δ60 ALP-based assay. The cells were treated with 200 ng/ml rapamycin for 0 (Log) or 4 hr (Rap) before analysis. (D) Atg13 and Atg17 were not required for the autophosphorylation of Atg1\textsuperscript{T226E} \textit{in vivo}. Cell extracts were prepared from either log-phase (L) or rapamycin-treated (R) cultures of wild-type, \textit{atg13}\textsuperscript{Δ}, and \textit{atg17}\textsuperscript{Δ} cells carrying the indicated Atg1 proteins. The relative levels of the slower-migrating, autophosphorylated form of Atg1 were assessed by Western blotting with an anti-myc antibody. The Atg1 proteins assayed were all tagged with three copies of the myc epitope.
Figure 2.10 Atg13 and Atg17 were required for Atg1 autophosphorylation within the activation loop.
Figure 2.11 Atg1 autophosphorylation resulted in an increase in kinase activity towards MBP.

(A) The stimulation of Atg1 kinase activity brought on by a pre-incubation with ATP required a functional Atg1 kinase domain. The indicated Atg1 proteins were immunoprecipitated from rapamycin-treated cells with an antibody specific for the myc epitope. The samples were then mock treated (−) or subjected to an autophosphorylation reaction with 3 mM ATP (+) for 60 mins at 30 °C. The samples were washed to remove the excess ATP and an \textit{in vitro} kinase assay was performed with [\gamma^{32}\text{P}] ATP and MBP. The reaction products were subsequently separated on an SDS-polyacrylamide gel and the relative level of MBP phosphorylation was assessed with a phosphorimager. The data shown are from a single experiment that was representative of three independent trials.

(B) A Western blot control showing the relative levels of Atg1 proteins present in the reactions described above in panel A was performed with an antibody specific for the myc epitope.
Figure 2.12 The Atg1$^{T226E}$ variant was competent for Atg1 autophosphorylation.

(A) The Atg1$^{T226E}$ variant was autophosphorylated in vitro. The indicated Atg1 proteins were immunoprecipitated from rapamycin-treated cells and subjected to an in vitro autophosphorylation assay as described in the Materials and Methods. The Atg1 proteins were tagged at the N-terminus with three copies of the myc epitope. (B) The slower-migrating form of Atg1 that was present in SDS-polyacrylamide gels following rapamycin treatment was due to autophosphorylation at a position distinct from T226. The indicated Atg1 proteins were precipitated from log phase cells, treated with $\lambda$ phosphatase and then incubated with 3 mM ATP. Atg1-P, the slower-migrating form of Atg1; PPase, $\lambda$ phosphatase; IVKA, in vitro kinase assay.
Figure 2.13 Phosphorylation of MBP by recombinant Atg1 proteins.

rAtg1 proteins with the indicated alterations were isolated from *E. coli* cells and incubated with MBP and [γ-32P] ATP. The reaction products were separated on SDS-polyacrylamide gels and the relative level of MBP phosphorylation was assessed with a phosphorimager. (−), control extract without any rAtg1 protein.
Figure 2.14 Assessing autophagy levels in wild-type cells over-expressing the Atg1T226A and Atg1T226E variants.

High-copy plasmids encoding the indicated Atg1 proteins were introduced into wild-type cells and autophagy levels were assessed with the Pho8Δ60 ALP-based assay as described in the MATERIALS AND METHODS. The cells analyzed were treated with 200 ng/ml rapamycin for 0 (Log) or 4 hr (Rap).
Figure 2.15 Atg13 and Atg17 were needed for Atg1 activation loop phosphorylation.

A darker exposure of the image presented in Fig. 10A is shown to highlight the low level of T226 phosphorylation detected in \textit{atg13Δ} and \textit{atg17Δ} cells. Note that no significant T226 phosphorylation was detectable in cells carrying only the kinase-defective variant, Atg1\textsubscript{K54A}. 
Chapter 3

The identification and analysis of phosphorylation sites on the Atg1 protein kinase

3.1 Introduction

Post-translational modifications are used to regulate many biological activities in response to changes in the environment. Autophagy is no exception. This process is tightly regulated by multiple protein kinases, including the Target of rapamycin (Tor), the cAMP-dependent protein kinase (PKA) and the AMP-activated protein kinase (AMPK) homolog, Snf1\(^{35,39,72-74}\). Several studies have shown that TORC1 phosphorylates Atg13 and thus interferes with Atg1 kinase activity and hence autophagy. PKA has also been shown to directly phosphorylate Atg1 and Atg13 to prevent autophagy\(^{73,92}\). Aspects of this regulation have been well conserved in other organisms, including mammals.

Atg1 is a serine/threonine-rich protein kinase and its kinase activity is required for the autophagy process\(^{86}\). Upon starvation, Atg1 protein kinase is significantly elevated. Atg1 was shown to be phosphorylated by several kinases, including TOC1, PKA and Atg1 itself, and these phosphorylation events control the autophagy pathway\(^{35,74,158}\). Our work has indicated that the activation loop phosphorylation of Atg1 is important for Atg1 kinase activity and the autophagy process\(^{158}\). In an effort to expand our understanding of the regulation of autophagy, we used mass spectrometry and the molecular biology approaches to systematically identify and analyze phosphorylation sites within Atg1.
3.2 Methods and Materials

3.2.1 Strains and growth media

Standard *E. coli* growth conditions and media were used in this study. The yeast rich growth medium, YPAD, consists of 2% glucose, 1% yeast extract, 2% Bacto-peptone and 500 mg/L adenine-HCl. The SC-glucose minimum growth medium has been described \(^{141,159}\). The yeast strains used were TN125 (*MATa ade2 his3 leu2 lys2 trp1 ura3 pho8::pho8Δ60*), YYK126 (TN125 *atg1Δ::LEU2*) and BY4741 (*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*) \(^{35,142,143}\).

3.2.2 Plasmid construction

Site-directed mutagenesis was performed with a gap-repair strategy \(^{160}\). The *ATG1*-containing plasmids, pPHY115 and pPHY2376, were digested with the restriction enzyme *Psh*AI, and then co-transformed into yeast cells with PCR products containing the desired mutations and flanking sequence complimentary to the gapped plasmid. The resulting plasmids were extracted and screened for the desired mutations. Plasmid pPHY1115 was originally named pRS316-3xmycATG1 and was generously provided by Dr. Yoshinori Ohsumi. Plasmid pPHY2376 is a pRS423-based construct where Atg1 has three copies of the HA epitope at its N-terminus; this plasmid was originally provided by Dr. Daniel Klionsky. The HA-tagged N-terminal Atg1 fragments were generated by a PCR reaction using pPHY2376 plasmid DNA as template and were cloned into the pRS423 vector, containing the *ATG1* promoter. The GST-Atg1 fusion protein was over-expressed from the *GAL1/10* promoter in a plasmid that was generously provided by Dr. Michael Snyder.
3.2.3 Autophagy assays

Autophagy activity was analyzed with three independent assays that have been described previously. In general, autophagy was induced by treating mid-log phase cells with 200 ng/ml rapamycin for the indicated times. The alkaline phosphatase-based assay measures the delivery and subsequent activation in the vacuole of a modified form of the Pho8 alkaline phosphatase, known as Pho8Δ60. The level of autophagy activity was determined by the difference in alkaline phosphatase activity detected between extracts prepared from log phase and rapamycin-treated cells. The data presented here were the average of at least three independent experiments. The Ape1 maturation assay assesses the processing of over-expressed aminopeptidase I, or Ape1, by the autophagy pathway. The elevated levels of Ape1 saturate the capacity of the Cvt system and result in the accumulation of the unprocessed precursor in the cytoplasm. The relative levels of the two forms of Ape1 were assessed by Western blotting with an anti-Ape1 antibody provided by Dr. Daniel Klionsky. In the GFP-Atg8 assay, the relative level of free GFP generated by proteolysis in the vacuole is used to indicate autophagy activity. The GFP-Atg8 fusion protein and the free GFP were detected by Western blotting with an anti-GFP antibody (Clontech).

3.2.4 Western blotting and immunoprecipitation

Protein samples were prepared as described. The proteins were separated on SDS-polyacrylamide gels, transferred to nitrocellulose membrane and then probed with the appropriate primary and secondary antibodies (GE Healthcare). A chemiluminescent substrate (Thermal Fisher Scientific Inc) or Odyssey infrared imaging system (LI-COR
Biosciences) was then used to detect the reactive bands or fluorescence intensities. Immunoprecipitations were performed as described \(^{74,161}\). HA-tagged proteins were precipitated on an anti-HA affinity matrix (Roche) whereas myc-tagged proteins were immunoprecipitated with a monoclonal anti-myc antibody (Cell Signaling) and subsequently collected on Protein A - Sepharose beads (GE Healthcare).

### 3.2.5 In vitro kinase assays (IVKAs)

After immunoprecipitation, Atg1 proteins were bound to beads and incubated with 10 μCi [γ-\(^{32}\)P] ATP or 2.5 mM cold ATP for 30 min in kinase buffer (25 mM MOPS, 1 mM EGTA, 100 μM Na\(_3\)VO\(_4\), 15 mM MgCl\(_2\) and 15 mM ρ-nitrophenylphosphate) \(^{35}\). The reaction products were separated by SDS–polyacrylamide gel electrophoresis and the relative level of radioactivity incorporated into the Atg1 proteins was assessed with a Typhoon Trio phosphorimager (GE Healthcare). The input level of each protein was assessed by Western blotting with the appropriate antibody.

### 3.2.6 Isolation of the GST-tagged Atg1 protein for the MS analysis

Yeast cells were grown to mid-log phase in an SC medium containing 2% raffinose. The induction of the GST-Atg1 fusion protein was induced by transferring the cells to a medium containing 5% galactose for 4 hrs \(^{162}\). The GST-Atg1 was immunoprecipitated, separated on an SDS-polyacrylamide gel and visualized by Coomassie G-250 staining (Bio-Rad). The GST-Atg1 band was then excised and used for the MS/MS analysis.
3.2.7 Tandem mass spectrometry

Coomassie Blue-stained protein bands were excised from gels, reduced and alkylated with iodoacetamide, and in-gel-digested with sequencing grade, modified trypsin (Promega). Peptides were extracted and subjected to capillary-liquid chromatography-nanospray tandem mass spectrometry (nanoLC-nanoESI-MS/MS) analysis for protein identification as described previously \(^{163}\).

3.3 Results

3.3.1 MS identification of potential phosphorylation sites on Atg1

To facilitate a more comprehensive mapping of Atg1 phosphorylation sites, phosphopeptides were enriched from the in-gel digested Atg1 tryptic peptide pool by the use of a TiO\(_2\) microcolumn. Both the original non-enriched sample and the TiO\(_2\)-bound fraction containing a majority of the phosphopeptides were subjected to several repeated runs of nanoLC-MS/MS analysis under data dependent acquisition mode and the resulting MS/MS datasets were individually searched against NCBInr database using the Mascot search engine. Combining the search results from all analyses, a sequence coverage of 67% was attained in total, which included confident identification of 14 unique mono-phosphorylated peptides (Figure 3.1, B–C). The corresponding MS/MS spectra for each of the tentatively assigned phosphorylation sites were further manually verified and annotated for the sequence informative b and y fragment ions (Appendix A). In general, phosphorylated site assignment was considered reliable if the b and y ions flanking the implicated site could be detected, with mass shifts corresponding to a phosphorylated serine or threonine, or one having eliminated its phosphate (- 98 Da).
Taken as an example, manual inspection of the MS/MS spectrum acquired on the triply charged precursor ion for peptide EIGKGSFATVYR at \( m/z \) 469.9 (Appendix A, Figure A1) showed that the mono-phosphorylated peptide was indeed phosphorylated exclusively at Ser34 and not Thr37. The y7 (pSFATVYR), y8 (GpSFATVYR) and y10 (GKGpSFATVYR) ions were detected exclusively as (y7-98), (y8-98) and (y10-98) ions at \( m/z \) 825.38 (1+), 882.36 (2+), and 534.27 (2+), respectively. In contrast, the y4 and y5 ions harboring the Thr37 site were detected without any phosphate or the loss of it at \( m/z \) 538.28 (1+) and 609.31 (1+), respectively. By such criteria, all of the assigned sites could be manually verified except the two sites at Ser-551 and Ser-552, carried on the mono-phosphorylated peptide 536-575, which could not be unambiguously resolved. Notably, the mono-phosphorylated peptide 675-688 was identified thrice, corresponding to the same peptide eluting at slightly different retention time and being phosphorylated at 3 different positions, i.e Ser-677, Ser-680 and Ser-683. Taken together, a total of 14 phosphorylation sites were confidently identified, 9 of which (Ser-34, Ser-304, Ser-533, Ser-551/552, Ser-621, Ser-680, Ser-769, Thr-129 and Thr-590) were not reported previously (Figure 3.1, B).

The phosphorylation sites identified on the \textit{S. cerevisiae} Atg1 were also compared with those detected recently on the mouse Ulk1 protein. Ulk1 is the mammalian protein kinase that exhibits the highest degree of sequence similarity to Atg1. Although the phosphorylation sites are distributed throughout both proteins in a similar manner, we did not detect any obvious overlap between the sites thus far identified. However, many of the phosphorylation sites occur within the less conserved, serine-rich central region of Atg1. Even if these sites were serving similar functional roles, it might be difficult to
discern this from sequence alignments alone. One of the identified sites, Ser-515 has been shown to be the site of PKA phosphorylation\(^7^4\). Several sites we found were overlapped with a large-scale phosphoproteome study, which looked for phosphorylated proteins in response to DNA damage in yeast *Saccharomyces cerevisiae* (Table 3.1)\(^1^6^5\). In all, we identified nine novel phosphorylation sites of Atg1.

### 3.3.2 Phosphorylation of serine 34 is a potential inhibition in the autophagy process

The MS/MS analysis identified Ser-34 as a site of phosphorylation within Atg1. This serine is found within a glycine-rich, GxGxxG motif (G-loop) that is conserved in many kinases (Figure 3.2, A)\(^1^6^6\). The best studied example of G-loop phosphorylation is that of the cyclin-dependent protein kinases (CDKs)\(^1^6^7^-1^6^9\). The phosphorylation of *Schizosaccharomyces pombe* Cdc2 on Thr-14 and Tyr-15 is inhibitory for cell cycle progression and this negative regulation has been conserved in other orthologs, including Cdc28 in *S. cerevisiae* and Cdk1 in humans. This inhibitory phosphorylation inactivates the mitotic activity of cyclin-dependent protein kinases and this prevents the entry into mitosis. The removal of this phosphorylation by Cdc25-like phosphatases is an important step for mitosis\(^1^7^0,1^7^1\). The location of Ser-34 led us to examine whether the phosphorylation is also inhibitory for autophagy. For these studies, we altered Ser-34 to either the nonphosphorylable alanine or the potential phosphomimetics, aspartic or glutamic acid. We then assessed whether these Atg1 variants could substitute the wild-type protein during autophagy. These phosphomimetic residues can successfully substitute for a phosphorylated serine due to their carboxylic acid moiety mimicking the negative charge of phosphate group\(^1^7^2,1^7^3\). We used two different assays to test whether
the phosphorylation at the Ser-34 of Atg1 might have an effect on autophagy. The first assessed the vacuolar delivery and processing of an altered form of the Pho8 alkaline phosphatase, known as Pho8Δ60 142. This truncated protein lacks a membrane-spanning domain and the targeting information necessary for normal delivery through the secretory pathway 142,149. As a result, this protein can be delivered to the vacuole, and therein activated, only by the autophagy pathway. Using this assay, we found that a wild-type level of autophagy activity was detectable in cells carrying only the Atg1S34A variant (Figure 3.2, B). In contrast, the levels of autophagy detected in cells containing either the Atg1S34D or Atg1S34E variant were similar to those associated with the atg1Δ control strain. We also replaced Ser-34 with a glycine, tyrosine and phenylalanine and then tested the effects of these substitutions on the induction of autophagy. Unlike the phosphomimetic variants, the levels of autophagy activity detected in these latter variants were considerably similar to the wild type (Figure 3.2, B).

The second assay used here measured the autophagy-dependent maturation of the Ape1 protein. Ape1 is a normal cargo for the Cvt, transport pathway that is responsible for delivering a set of proteins to the vacuole during log-phase growth 32,150. Overexpression of Ape1 saturates the Cvt system and results in the cytoplasmic accumulation of an unprocessed, precursor form of this protein. Upon the induction of autophagy, this protein is efficiently delivered to the vacuole and processed to its mature, active form 79,151. We found that the Atg1S34D variant was defective for this autophagy-mediated processing and that the Ape1 protein was present only in its precursor form in these cells. However, the Atg1S34A variant was normal for Ape1 maturation (Figure 3.2, C). Altogether, these data are consistent with the phosphorylation at Ser34 in Atg1, being
inhibitory for the autophagy pathway in *S. cerevisiae*.

### 3.3.3 Phosphorylation of Ser-34 inhibits Atg1 kinase activity

We also examined the kinase activity associated with the above Ser-34 variants. To assess Atg1 kinase activity *in vivo*, we took advantage of previous observations indicating that Atg1 autophosphorylation results in the production of a slower-migrating form of this protein on SDS-polyacrylamide gels (Atg1-P)\(^{73,158}\). The relative level of this second, upper band can serve as a measure of the Atg1 kinase activity present in cells. Compared to the wild type, we found significantly less of the Atg1\(^{S34E}\) variant in the slower migrating form (Atg1-P), following rapamycin treatment (Figure 3.3, D). In contrast, we detected similar levels of this upper band with the Atg1\(^{S34A}\) variant. We can also use this gel-based assay to assess Atg1 kinase activity *in vitro*\(^{158}\). Immunoprecipitated Atg1 proteins were treated with λ phosphatase, incubated with ATP, separated on SDS-polyacrylamide gels and analyzed by Western blotting with appropriate antibodies. As described above, relative amount of Atg1 present in the slower-migrating form would be a measure of Atg1 kinase activity. Consistent with the *in vivo* results, we found that the Atg1\(^{S34A}\) variant resembled the wild-type protein whereas both Atg1\(^{S34D}\) and Atg1\(^{S34E}\) variants exhibited less activity. In all, these data suggested that the phosphorylation of Ser-34 in Atg1 inhibits both its kinase activity and the autophagy process.
3.3.4 Analysis of identified phosphorylation sites

We identified fifteen phosphorylation sites in total with the tandem mass spectrometry. To understand the contribution of each phosphorylation site to both Atg1 kinase activity and autophagy pathway, we changed each serine (or threonine) to the nonphosphorylated residue, alanine, in the full-length Atg1 protein and examined whether these Atg1 serine-to-alanine variants were functional for autophagy (Figure 3.3, A). Using the ALP-based assay described above, we found that a few variants, including Atg1S551A, Atg1S552A, Atg1S621A, Atg1S677A, Atg1S683A and Atg1S783A exhibited a 30% – 40% reduction of autophagy activity relative to the wild type.

We also assessed Atg1 kinase activity both in vivo and in vitro. For the in vivo activity, we used the Western blot-based assay described above. For the in vitro assays, we assessed autophosphorylation in vitro with γ-32P [ATP]. We found that all of the altered proteins exhibited autophosphorylation activity in vivo based on the presence of the slower-migrating form of Atg1 in rapamycin-treated cells (Figure 3.3, B). All of the variants tested also exhibited kinase activity in an in vitro autophosphorylation assay (Figure 3.3, C). The greatest defects were observed with the Atg1S621A variant that had only 50% the activity of the wild-type and the Atg1S680A variant that reproducibly exhibited more activity than the wild-type protein. This latter result was interesting in light of the autophagy data above where the presence of the S680A alteration led to increased activity in a protein that already possessed the two changes, S677A and S683A (Figure 3.3, A).

In summary, about half of the single alterations reduced autophagy by 25 to 40% and therefore these sites may be important for the normal control of this degradative
response *in vivo*. In addition, there might be some level of cooperation amongst these phosphorylation sites that we have not yet uncovered. The appropriate combination of alterations, perhaps including some sites that have not yet been identified, could result in a stronger effect on the autophagy process. Finally, it is important to point out that we have only looked at macroautophagy here and that some of these alterations might have a greater effect upon other types of autophagy processes.

**3.3.5 Ser-390 is a site of autophosphorylation, responsible for the slower-migrating form of Atg1 observed on SDS-polyacrylamide gels**

The presence of the slower-migrating form of Atg1 in SDS-polyacrylamide gels requires a functional Atg1 kinase domain and is lost upon phosphatase treatment \(^{73,158}\). These and other results suggested that this anomalous migration was due to a specific autophosphorylation event. However, the phosphorylation site responsible for this shift has not yet been identified. All of the alanine variants analyzed above exhibited the characteristic doublet for Atg1 when analyzed by gel electrophoresis. Therefore, we used a combined deletion and mutagenesis approach here to identify this autophosphorylation site. Since the kinase domain is located at the very N-terminus of Atg1, we examined the autophosphorylation of Atg1 fragments with increasingly larger deletions from the C-terminus. We found that fragments containing the N-terminal 420 (Atg11-420) or 450 (Atg11-450) amino acids were capable of autophosphorylation *in vitro* (Figure 3.4, A). Shorter fragments, including the Atg11-384 construct, were nonfunctional in this assay. The Atg1 kinase domain is known to contain at least one site of autophosphorylation at Thr-226 within the activation loop \(^{158}\). To test whether the observed incorporation of
radioactivity into the Atg11-420 fragment was due solely to this site, we took advantage of observations from a previous analysis of this phosphorylation event. These studies had shown that an Atg1 variant containing a phosphomimetic at position 226, such as Atg1\textsuperscript{T226E}, was able to undergo an autophosphorylation reaction \textit{in vitro}\textsuperscript{158}. Therefore, we asked whether the Atg11-420 fragment would still incorporate radioactivity when Thr-226 was replaced with a glutamic acid. We found that the T226E version of this fragment was still functional in this \textit{in vitro} assay suggesting that there was a second site of autophosphorylation within the N-terminal 420 amino acids of Atg1 (Figure 3.4, B).

To map this site, we set out to systematically replace the serine and threonine residues present within this fragment with an alanine. This procedure was initiated from position 420 and moved progressively towards the N-terminus of the protein. We found that the replacement of Ser-390 in the full-length protein, but none of the other residues tested, resulted in the loss of the slower migrating form of Atg1 (Figure 3.4, C–D). In addition, replacing this residue with the potential phosphomimetics, aspartic or glutamic acid, resulted in an Atg1 protein that migrated only as the upper band on SDS-polyacrylamide gels (Figure 3.4, C–D). These data therefore suggested that Ser-390 might be the site of phosphorylation responsible for the band shift observed with active Atg1. However, the absence of the slower-migrating form of the Atg1\textsuperscript{S390A} variant could also have been due to a loss of kinase activity. To test this possibility, we carried out \textit{in vitro} autophosphorylation assays with the Atg1\textsuperscript{S390A} and Atg1\textsuperscript{S390D} variants. These variants exhibited similar levels of kinase activity that was somewhat less than that seen with the wild-type protein (Figure 3.5, A). This decrease was not unexpected as these variants would be predicted to have one fewer site of autophosphorylation. As a second
measure of kinase activity, we asked whether these S390 variants were capable of autophosphorylation at Thr-226. For this analysis, we used an antibody that specifically recognizes the phosphorylated form of this position and found that each of these variants was indeed phosphorylated at Thr-226 (Figure 3.5, B). In all, these data suggested that the phosphorylation at Ser-390 is both necessary and sufficient for the presence of the slower-migrating form of Atg1 detected on SDS-polyacrylamide gels. Although our MS analysis here did not identify Ser-390 as a site of phosphorylation, this modification was detected in a previous large-scale analysis of the yeast phosphoproteome (Table 3.1) 165. 

Finally, we tested whether these alterations at Ser-390 affected the autophagy process. For these experiments, we used the Pho8Δ60 and Ape1 assays described above as well as a third assay that assesses the processing of an Atg8-GFP fusion protein. This fusion is delivered to the vacuole via the autophagy pathway and the Atg8 moiety is then degraded by the hydrolases present within this compartment 56. GFP is more resistant to this degradation and the relative level of the free GFP that accumulates has been used as a measure of autophagy activity 56. We found that cells containing either the Atg1S390A or Atg1S390D variant exhibited autophagy activity in each of these three assays (Figure 3.5, C–E). These data therefore suggested that the phosphorylation at Ser-390 does not significantly influence the macroautophagy process induced by rapamycin treatment. However, as with the other sites identified here, it will be important to assess whether this modification has a role in other selective autophagy processes 30,174-177.

3.4 Discussion

In this study, we identified sixteen phosphorylation sites on the S. cerevisiae Atg1
protein, nine of which had not been identified previously. The functional analysis indicated that the phosphorylation at one of these sites, Ser-34 in the conserved glycine-rich loop of the kinase domain, might be inhibitory for Atg1 kinase activity and the autophagy process. Future work will be directed at identifying the protein kinase responsible for this modification and the physiological significance of this inhibition. Mutation of any other individual phosphorylation site did not totally abolish autophagy activity. However, these phosphorylation sites may have different levels of cooperation in control of autophagy, which we have not explored. For example, a recent study has shown that the overexpression of an Atg13 that harbors eight Ser-to-Ala mutations, bypasses the TORC1 pathway to induce autophagy in the nutrient-rich conditions 92. Moreover, Atg1 is essential for all types of autophagy and these identified phosphorylation sites of Atg1 may have an uncovered role in one of the other pathways.

We also identified the phosphorylation site, Ser-390 is responsible for the mobility shift on SDS-polyacrylamide gels. This shift has been used routinely to assay Atg1 kinase activity in vivo. This upper-shift protein band was detected from the cells treated with rapamycin and considered as the active form of Atg1, implicating the potential conformational change in the structure of the active Atg1 protein kinase. Some particular phosphorylation is known to alter the protein conformation for the induction or modulation of its physiological activity, such as the conserved activation loop phosphorylation in most protein kinases 97,178. Further work is needed to examine the phosphorylation-induced structure change of Atg1 in the nutrient-limited condition. In all, the phosphorylation sites identified here provide a framework of the potential avenues for the autophagy regulation that can now to be explored in future work.
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Table 3.1 A comparison of the phosphorylation sites identified in the *S. cerevisiae* Atg1.
The first column lists the sites identified in the MS/MS analysis performed here, the second column the sites identified in a previous study and the third column sites that were identified by other means 74,158,165. The sites highlighted were identified in only one of the studies summarized here.
Figure 3.1 A phosphorylation site map for the *S. cerevisiae* Atg1.

(A) The phosphorylation sites in Atg1 that were identified in this study. The sixteen sites identified herein are indicated above the Atg1 schematic. All were identified by the MS analysis except for S390 (indicated by a double asterisk). S515, indicated by a single asterisk, was identified previously as a site of PKA phosphorylation. The two positions indicated below the Atg1 schematic were identified previously: S508 was also found to be a site of PKA phosphorylation and T226 is a conserved site of autophosphorylation within the Atg1 kinase domain. The conserved kinase and C-terminal domains of Atg1 are indicated with the light gray (KINASE) and dark gray (CTD) shading, respectively. (B) The list of tryptic phosphopeptides identified by the tandem MS/MS analysis. The site of phosphorylation in each peptide is shown in bold face. (C) The peptide coverage of Atg1 protein is 67%. The mass spectrometry (MS) detected peptides are underlined and the site of phosphorylation is highlighted in gray.
Figure 3.1 A phosphorylation site map of the *S. cerevisiae* Atg1.
Figure 3.2 Phosphorylation at Ser-34 within Atg1 is a potential site of inhibition for the autophagy process.

(A) A sequence alignment of the glycine-rich loop (G-loop) present in Atg1 and the mammalian Cdc2 enzymes. The consensus sequence for this motif is shown at the top and the residues known to be phosphorylated are underlined. (B) Assessing autophagy with the Pho8Δ60 alkaline phosphatase-based assay. The relative levels of autophagy for an atg1Δ strain carrying single-copy plasmids expressing the indicated variants of Atg1 are shown. Autophagy was induced by treating log phase cultures with 200 ng/ml rapamycin for 4 hr. The data shown are the average of at least three independent experiments and the error bars indicate one standard deviation. (C) Autophagy activity as measured with the Ape1 processing assay. Cells over-expressing Ape1 were treated with 200 ng/ml rapamycin for the time indicated to induce the autophagy process. The relative level of the mature, processed form of the Ape1 protein (mApe1) was an indicator of the autophagy activity present. prApe1, precursor Ape1. The bottom panel shows the relative levels of the different myc-tagged Atg1 proteins in the strains examined. (D) An analysis of Atg1 autophosphorylation activity in vivo. The relative level of the autophosphorylated form of Atg1 (Atg1-P) was assessed in atg1Δ cells expressing the indicated variants of Atg1. Western blots were performed with extracts prepared from either log phase (L) or rapamycin-treated (R) cells. (E) An examination of the effects of altering Ser-34 on Atg1 autophosphorylation in vitro. The indicated myc epitope-tagged Atg1 proteins were immunoprecipitated and then either mock treated (IP) or incubated with 1 phosphatase (PPase). An aliquot of the phosphatase-treated protein was then subjected to an in vitro autophosphorylation reaction (IVKA) with 2.5 mM ATP, as described in the Materials and Methods. The resulting reaction products were separated by SDS-polyacrylamide gel electrophoresis and the relative level of the autophosphorylated form of Atg1, Atg1-P, was assessed by Western blotting. The A, D and E designations indicate the Atg1S34A, Atg1S34D and Atg1S34E proteins, respectively.
Figure 3.2 Phosphorylation at Ser-34 within Atg1 is a potential site of inhibition for the autophagy process.
Figure 3.3 An analysis of the autophagy and kinase activities associated with alterations of the Atg1 phosphorylation sites identified in this study.

(A) Autophagy activity as measured with the Pho8D60 alkaline phosphatase-based assay. The relative levels of autophagy present in an atg1Δ strain expressing the indicated Atg1 protein variants are shown. Autophagy was induced by treating log phase cultures with 200 ng/ml rapamycin for 4 hr. The values are shown as a percentage of the activity observed with the wild-type Atg1. The data shown are the average of at least three independent experiments and the error bars indicate one standard deviation. (B) An assessment of Atg1 autophosphorylation levels in vivo. The relative levels of the autophosphorylated form (Atg1-P) of each of the indicated Atg1 variants was assessed by Western blotting. The extracts were prepared from either log phase (L) or rapamycin-treated (R) cells. (C) Atg1 autophosphorylation in vitro. The indicated Atg1 proteins were immunoprecipitated from rapamycin-treated cells and subjected to an in vitro autophosphorylation assay with [γ-32P] ATP, as described in the MATERIALS AND METHODS. The reaction products were separated on an SDS-polyacrylamide gel and the relative amount of radioactivity incorporated into Atg1 was assessed with a phosphorimager. The data presented represent the average of at least two independent experiments where the phosphorylation levels varied by less than 15% between the different experimental replicates.
Figure 3.3 An analysis of the autophagy and kinase activities associated with alterations of the Atg1 phosphorylation sites identified in this study.
Figure 3.4 Autophosphorylation at Ser-390 is responsible for the slower-migrating form of Atg1 observed on SDS-polyacrylamide gels.

(A) An in vitro autophosphorylation assay with N-terminal fragments of Atg1. The indicated Atg1 deletion constructs were immunoprecipitated from yeast cell extracts, incubated with \( \gamma^{32}\text{P} \) ATP and then separated on SDS-polyacrylamide gels. The relative amount of radioactivity incorporated into each Atg1 fragment was assessed by autoradiography. The lower panel shows a Western blot depicting the relative amount of each construct present in the kinase reactions. The positions of the relevant protein bands are indicated with an asterisk. (B) The identification of a second site of autophosphorylation within the N-terminal 420 amino acids of Atg1. Atg11-420 fragments with either a threonine (Wt) or glutamic acid (T226E) at position 226 were precipitated from cell extracts and then subjected to an in vitro autophosphorylation assay with \( \gamma^{32}\text{P} \) ATP, as described in the Materials and Methods. The relative amount of radioactivity incorporated into each fragment was determined by autoradiography. The lower panel shows a Western blot depicting the relative amount of each construct present in the kinase reactions. (C) Alterations of Ser-390 influence Atg1 mobility in an SDS-polyacrylamide gel. Western blots were performed with extracts prepared from either log phase (L) or rapamycin-treated (R) cells expressing the indicated variants of Atg1. D211A, a kinase-defective Atg1. (D) The gel mobility of the Atg1\(^{S390A}\) and Atg1\(^{S390D}\) variants were not affected by either a phosphatase treatment or autophosphorylation reaction. The indicated Atg1 proteins were immunoprecipitated and then either mock treated (IP) or incubated with 1 phosphatase (PPase). An aliquot of the phosphatase-treated protein was then subjected to an in vitro autophosphorylation reaction (IVKA) with 2.5 mM ATP, as described in the Materials and Methods. The resulting reaction products were separated by SDS-polyacrylamide gel electrophoresis and the relative mobility on SDS-polyacrylamide gels was assessed by Western blotting. The A and D designations indicate the Atg1\(^{S390A}\) and Atg1\(^{S390D}\) proteins, respectively.
Figure 3.4 Autophosphorylation at Ser-390 is responsible for the slower-migrating form of Atg1 observed on SDS-polyacrylamide gels.
Figure 3.5 Protein kinase and autophagy activities associated with alterations of Ser-390 in Atg1.

(A) Atg1 autophosphorylation in vitro. The indicated Atg1 proteins were immunoprecipitated from rapamycin-treated cells and subjected to an in vitro autophosphorylation assay with $\gamma^{-32}P$ ATP, as described in the Materials and Methods. The reaction products were separated on an SDS-polyacrylamide gel and the relative amount of radioactivity incorporated into Atg1 was assessed with a phosphorimager. The data presented represent the average of at least two independent experiments where the phosphorylation levels varied by less than 10% between the different experimental replicates. (B) Assessing the levels of Thr-226 phosphorylation in vivo. The indicated Atg1 proteins were immunoprecipitated from rapamycin-treated cells, separated on SDS-polyacrylamide gels, and then analyzed by Western blotting with the indicated antibodies. The protein signals were detected with a LI-COR Odyssey infrared detection system. The relative signal detected with the anti-pT226 phosphospecific antibody ($\alpha$-pT226) is a measure of the level of autophosphorylation at Thr-226. The A, D and E designations indicate the Atg1$^{S390A}$, Atg1$^{S390D}$ and Atg1$^{S390E}$ proteins, respectively. (C–E) Autophagy activity associated with atg1D cells expressing the indicated Atg1 proteins was assessed with the Pho8D60 processing (in C), Ape1 maturation (in D) and GFP-Atg8 processing (in E) assays, as described in the MATERIALS AND METHODS. Autophagy was induced by treating log phase cells with 200 ng/ml rapamycin for 4 hrs (in C) or the times indicated (in D and E).
Figure 3.5 Protein kinase and autophagy activities associated with alterations of Ser-390 in Atg1.
Chapter 4

Investigation of the role of Atg13 in promoting Atg1 kinase activity and the autophagy process

4.1 Introduction

Most of the molecular components of the autophagy pathway were initially characterized in the budding yeast, *S. cerevisiae*, but orthologs of many of these Atg proteins have since been found in other eukaryotes. A complex of proteins that contains the Atg1 protein kinase is of special interest and appears to be a key point of regulatory control within this pathway. Genetic and biochemical data suggest that this complex is the major regulatory target of at least two different signaling pathways in *S. cerevisiae*, involving the Tor and cAMP-dependent protein kinases, inhibit this process, whereas the AMP-activated protein kinase pathway is needed for the full induction of autophagy. The manner in which these pathways regulate Atg1 activity, and the precise role of this kinase in the autophagy process, are presently matters of intense scrutiny.

Although Atg1 kinase activity is required for the induction of autophagy, relatively little is known about how this enzyme is regulated *in vivo*. Two proteins associated with Atg1, Atg13 and Atg17, have been shown to be required for full Atg1 kinase activity *in vitro*. The roles of these proteins appear to be conserved through evolution as functional homologs of both have been identified in fruit flies and/or
mammals. However, it is not yet clear precisely how these proteins stimulate Atg1 activity. In the previous chapter, we have shown that Atg1 is autophosphorylated within the activation loop and this phosphorylation is required for both kinase activity and the induction of autophagy. The activation loop is a structurally conserved element within the kinase domain and phosphorylation within this loop is often a necessary prerequisite for efficient substrate binding and/or phosphotransfer in the catalytic site. Interestingly, our data suggest that promoting this autophosphorylation may be a primary role of Atg13 in the autophagy pathway. A model describing the activation of Atg1 and the implications in the regulation of autophagy in other eukaryotic organisms is discussed.

4.2 Methods and Materials

4.2.1 Strains and growth media

Standard E. coli growth conditions and media were used in this study. The yeast rich growth medium, YPAD, consists of 2% glucose, 1% yeast extract, 2% Bacto-peptone and 500 mg/L adenine-HCl. The SC-glucose minimum growth medium has been described. The yeast strains used were TN125 (MATa ade2 his3 leu2 lys2 trp1 ura3 pho8::pho8Δ60), YYK126 (TN125 atg1Δ::LEU2), YYK130 (TN125 atg13Δ::TRP1), BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and PHY4181 (BY4741 atg17Δ::KanMX).

4.2.2 Plasmid Constructions

pPHY1115 contained a C-terminal, 3x Myc-tagged full-length Atg1 protein under ATG1 promoter and pPHY2376 was composed of a N-terminal, 3x HA-tagged full-length Atg1
protein under its own promoter. These plasmids were generously provided by Dr. Yoshinori Ohsumi and Dr. Daniel Klionsky. To construct the \textit{ATG1} deletions, the appropriate fragments were PCR amplified from plasmid pPHY115 and cloned into pRS424 with C-terminal 3xMyc epitope-tag. The plasmids pPHY2426 and pPHY2806 consist of Atg13 and Atg17 respectively as described previously 179. The C-terminal Atg1 fragments were generated by PCR-amplification using pPHY1115 as the template and cloned into pRS424 vectors with 3x Myc-epitope at carboxyl terminus under \textit{ATG1} promoter. Plasmids used in this study were listed in Table 4.1.

4.2.3 Autophagy assay

Autophagy activity was assessed with the ALP-based assay that measures the delivery and subsequent activation in the vacuole of an altered form of the Pho8 phosphatase, Pho8Δ60 142. Pho8 is dependent on the membrane-spanning region to target the vacuole and be catalytically activated 145,151. Pho8Δ60 lacks the N-terminal 60 amino acids including the membrane-spanning regions and remains catalytically inactive form in the cytosol. This modified version, Pho8Δ60 requires the autophagy process for the delivery to the vacuole. Thus, the phosphatase activity of Pho8Δ60 detected in cell extracts can be used as a measure of the levels of the autophagy activity.

4.2.4 Immunoprecipitations

HA-tagged proteins were generally precipitated on anti-HA matrix agarose (Roche) as described 74. The amount of immunoprecipitated and co-immunoprecipitated proteins were separated on SDS-polyacrylamide gels and subsequently assessed by Western
blotting with the appropriate antibodies or by the Odyssey infrared imaging system (LI-COR Biosciences)\textsuperscript{73,158}.

4.3 Results

4.3.1 Determination of Atg13 interaction domain (TID) at the carboxyl-terminus of Atg1

Atg13 has been shown to be important for the Atg1 kinase activity\textsuperscript{35,158} and other work indicated that Atg13 might interact with Atg1 through the carboxyl-terminal domain of Atg1\textsuperscript{50,97}. In order to better understanding the role of Atg13 in the activation of Atg1, we examined the interactions between both proteins. We generated a series of Atg1 deletion constructs and analyzed the ability of these fragments to bind to Atg13 by co-immunoprecipitation assays (Figure 4.1). Using these constructs, we found that the C-terminal region of Atg1 was sufficient for the interaction with Atg13 (Figure 4.4, B–C). A relatively strong interaction was observed with an Atg1 construct of 348 amino acids (a.a. 550 – C) but weak binding was detectable with a smaller fragment that contained 247 residues (a.a. 650 – C). These observations were consistent with previous work showing that alterations within this C-terminal domain disrupted the interaction with Atg13\textsuperscript{50}. Moreover, recent studies with the corresponding mammalian proteins found that a similar region of mammalian Atg1 ortholog, Ulk1 was responsible for the association with mammalian Atg13\textsuperscript{46}. We will refer to this C-terminal region of Atg1 as the thirteen interaction domain, or TID, for the remainder of this chapter.
4.3.2 Atg13 and Atg17 play distinct roles in stimulating the Atg1 kinase activity

Atg13 and Atg17 are required for Atg1 kinase activity and our previous observations demonstrated that both proteins were also necessary for the activation loop phosphorylation of Atg1. However, deletion of either protein caused different levels of reduction of the Atg1 kinase activity measured by *in vitro* kinase assay. It is suggested that Atg13 and Atg17 might function differently in stimulating the Atg1 kinase activity. Our lab and others have shown that deletion of either protein resulted in loss of the Atg1 kinase activity *in vivo* and *in vitro*. Here, we further tested whether the over-expression of Atg13 or Atg17 would have any impact on the autophagy pathway. As expected from our previous work, the over-expression of Atg13 resulted in elevated autophagy upon rapamycin treatment. In contrast, elevated levels of Atg17 attenuated the level of autophagy activity in the Pho8Δ60-based assays (Figure 4.2, A). We also examined the effects of this over-expression on the induction of Atg1 kinase activity, which was measured here by the relative levels of Atg1 activation loop phosphorylation (Figure 4.2, B). We found that the Atg1 kinase activity was enhanced with the over-expression of Atg13, but diminished upon Atg17 over-expression. In all, these results suggest that Atg13 and Atg17 might work differently in the activation of Atg1 kinase activity.

4.3.3 Atg1-Atg13 interaction is important for autophagy activity

Several studies have shown that the Atg1 protein complex is the target of regulation by multiple signaling pathways. The Atg1 protein complex consists of Atg1, Atg13 and Atg17, and the precise interaction between these proteins has been suggested to be
critical for the proper control of autophagy\textsuperscript{35,50,51,73,74}. Therefore we tested here whether down-regulation of the Atg1-Atg13 interaction would affect the autophagy process. We interfered with Atg1-Atg13 interaction binding by adding an excess of Atg1 TID-containing fragments to unbalance the stoichiometry of the Atg1 protein complex. We assessed Atg1 kinase and autophagy under these conditions. If these Atg1 fragments contained the TID, we found that the over-expression resulted in reduced levels of activation loop phosphorylation (Figure 4.2, D). The overexpression of fragments that did not contain the TID had no significant effect on the Thr-226 phosphorylation level present. Similarly, over-expression of the TID-containing fragments of Atg1 led to a decreased level of autophagy in wild-type cells (Figure 4.2, C). In general, the interfering effects of the Atg1 fragments in these assays were correlated to the relative affinity each exhibited for Atg13. In all, these data suggested that Atg1-Atg13 interaction was important for both Thr-226 phosphorylation in Atg1 and the induction of autophagy.

4.3.4 An Atg1-Atg1 interaction requires the presence of Atg13

During the course of these studies, we found that Atg1 also was able to self-associate (Figure 4.3). This Atg1-Atg1 interaction was readily detected with full-length proteins in a co-immunoprecipitation assay. For these assays, we expressed two differently-tagged versions of respective Atg1 fragments. In an attempt to identify the domain responsible for this interaction, we divided Atg1 into two fragments: a 330 residue N-terminal region that contained the kinase domain (KIN) and a C-terminal fragment from 331 – 897 (CTerm). We observed a weak interaction between the full-length protein and the KIN fragment but no interaction with the CTerm domain (Figure 4.3, B). In addition, a very
weak, but detectable, interaction was noted between two KIN domains (Figure 4.3, C). Finally, no binding was observed between two CTerm fragments (Figure 4.3, C). Based on a series of dilutions, we estimate that there was 50-times less KIN fragment bound than the full-length in our co-immunoprecipitation assays with a second full-length protein. These assays were therefore consistent with a requirement for both N- and C-terminal element in the Atg1-Atg1 interaction.

We then asked if Atg13 or Atg17 was needed for the Atg1 self-association. Our data showed that the presence of Atg13 was indeed required for this Atg1-Atg1 interaction; the extent of self-association was diminished in atg13Δ mutants and was elevated in cells over-expressing Atg13 (Figure 4.4). However, these effects were specific to Atg13 as the Atg1-Atg1 interaction was largely unaffected by the absence or the over-expression of Atg17 (Figure 4.4).

We next tested whether the Atg1-Atg13 interaction might be important for the observed Atg1 self-association. For this analysis, we once again used the over-expression of the Atg1 TID-domain to disrupt Atg1-Atg13 association. Our goal was to test if the disruption of this latter interaction led to a loss of performed Atg1-Atg1 complexes. We found that Atg1 self-association was disrupted specifically by the presence of Atg1 fragments that contained TID (Figure 4.5, A). In contrast, the over-expression of the KIN domain had no effect on this self-association. The extent of the disruption was dose-dependent and proportional to the binding affinity exhibited by the Atg1 fragments to Atg13. (Figure 4.5, B–C). Therefore, these data suggested that Atg13 promoted and stabilized this Atg1 self-association.
4.3.5 Atg1\textsuperscript{T226E} variant formed oligomers in the Atg13-independent fashion

Atg13 is required for Atg1 kinase activity and the activation loop phosphorylation that is a prerequisite for the full activation of Atg1\textsuperscript{158}. Altogether, these results suggest a potential link between the activation loop phosphorylation and Atg1 self-association and that this link might be Atg13. One possibility is that Atg13 might facilitate Atg1 self-association and thus promotes the activation loop phosphorylation within Atg1. One mechanism for activation loop phosphorylation involves dimer formation between two unphosphorylated kinase molecules\textsuperscript{107,155,180,181}. Each protein in the dimer can then catalyze the cross-phosphorylation of its partner in the activation loop. The kinase activity is thus activated as a dimer. Here, we tested how replacing T226 in the activation loop with the phosphomimetic, glutamic acid (Atg1\textsuperscript{T226E}), would affect Atg1 self-association and its dependence upon Atg13. In contrast to the wild type Atg1, we found that the Atg1\textsuperscript{T226E} variant was able to self-associated with or without the presence of Atg13 (Figure 4.6, A). A role for Atg13 in promoting Atg1 kinase activity and self-association is proposed in Figure 4.6 B and discussed in detail in the DISCUSSION.

4.4 Discussion

We have shown that Atg1 and Atg13 associated with Atg1 via a carboxyl-terminal domain of the latter \textit{in vivo}. This Atg13 interaction domain (TID) is similar to that identified for ULK1, the mammalian Atg1 ortholog\textsuperscript{97}. The Atg1-Atg13 interaction was demonstrated to be important for Atg1 kinase activation and hence autophagy function in this study. Two conserved regulators of Atg1 activity, Atg13 and Atg17, were shown to be required for the Atg1 kinase activity and autophagy, but each appeared to be acting in
a mechanistically different manner. Our data also suggested that the primary role of Atg13 might be stimulating this autophosphorylation at Thr-226 and promoting a specific Atg1-Atg1 interaction in vivo.

Many protein kinases require phosphorylation within the activation loop for full activity. However, for those instances where this modification is self-catalyzed, it is unclear how this event takes place. The central question is how the first enzyme would be modified in the absence of any pre-existing active molecules. A number of possibilities have been suggested but one model is especially interesting in light of the data obtained here with Atg1. This model suggests a mechanism of kinase activation that involves the exchange of activation loops between members of a dimer complex. This exchange was initially described for the human checkpoint kinase 2, Chk2. In these studies, Chk2 was crystallized in an active state as a dimer where the activation loop from each subunit was interacting with the catalytic domain of the second kinase molecule. This model has since been found to apply to several additional protein kinases (Pike et al., 2008). The data here are consistent with a similar activation step for Atg1, where Atg13 would function to stabilize a dimer, or higher-order state, of Atg1 and thereby allow for autophosphorylation within the activation loop (Figure 4.6, B). In addition, previous studies have shown that the requirement for Atg13 can be bypassed by over-expressing Atg1. This over-expression may promote the formation of Atg1 dimers by mass action even in the absence of Atg13. It is also supported by our results that the recombinant Atg1 retained kinase activity as measured by the in vitro autophosphorylation. Atg13 and Atg17 are both key modulators of Atg1 kinase activity although the function of both proteins is not yet clear. Here, our biochemical data showed that overexpression of Atg17
had an inhibitory effect on Atg1 kinase and autophagy activities, suggesting that Atg17 may play a fundamentally different role from Atg13. However, this possibility requires further investigation to conclude the distinctly physiological function of Atg17 in the autophagy process.

Finally, we showed here that the Atg1-Atg13 association was required for Atg1 activation and self-association. Overexpression of carboxyl-terminal Atg1 fragments (for example, CTerm) that contained the TID interrupted this interaction and diminished Atg1 kinase and autophagy activities. Interestingly, the Atg1T226E variant could bypass the need for Atg13 for both the self-association and autophosphorylation at Ser-390\(^{158}\). In this model, Atg13 binding could induce a conformational change that allows the T-loop phosphorylation to occur through the Atg1 kinase domains, thus forming the stable Atg1 oligomers. This possibility could explain why we found that C-terminal fragments of Atg1 that contain the TID (for example, 331-C) did not interact with the full-length Atg1 protein. Alternatively, the N-terminal protein kinase domain and the C-terminal TID might bind cooperatively and thus both would be required for stabilizing Atg1 self-association. In all, the data here suggest that one of the primary functions of Atg13 during the induction of autophagy is the stimulation of Atg1 autophosphorylation within its activation loop by promoting a specific Atg1-Atg1 interaction \textit{in vivo}. Since Atg1 and its regulators are conserved through evolution, a continued analysis of the precise control of Atg1 protein kinase is likely to provide important insights into the regulation of a process relevant to human health.
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Table 4.1 List of plasmids used in this study.
Figure 4.1 Atg13 interacts with the carboxyl-terminus of Atg1.

(A) The scheme shows myc-tagged, amino-terminal truncated fragments used in this study to determine the Atg13-interaction domain (TID) of Atg1. The kinase domain indicated as “KINASE” contains amino acids 1 – 330 of Atg1. The number represents the position of amino acid in the Atg1 protein sequence. (B - C) Identifying the domain of Atg1 that was responsible for the interaction with Atg13. The indicated fragment of Atg1 was co-expressed in cells with Atg13. The full-length Atg1 is 897 residues. The Atg13 was precipitated with anti-HA agarose and the relative amount of the associated Atg1 fragments was assessed by Western blotting. The bottom panel of B and C showed the total amount of Atg1 fragments present in cells measured by Western blotting. (CTerm, 331 – 897 fragment of Atg1; Asterisk, Fragment1 – 529; Bullet, degradation bands.)
Figure 4.1 Atg13 interacts with the carboxyl-terminus of Atg1.
Figure 4.2 Atg13 and Atg17 played a distinct role in regulating the Atg1 kinase and the autophagy activities.

(A) Overexpressed Atg13 resulted in the high level of autophagy activity. In contrast, overexpressed Atg17 led to the reduced autophagy activity. Autophagy levels were assessed with the ALP Pho8Δ60-based assay in rapamycin-treated treated cell extracts with the overexpressed Atg13 or Atg17. The atg1Δ control strain contained a vector plasmid. (B) Overexpression of Atg13 or Atg17 had the opposite impact on the Atg1 kinase activity. The levels of Thr-226 phosphorylation were used to examine Atg1 kinase activity. The full-length Atg1 protein was immunoprecipitated from cells containing overexpressed Atg13 or Atg17, separated on the SDS-polyacrylamide gels and detected by Western blotting with the phospho-specific antibodies against Thr-226 phosphorylation within Atg1. (C - D) The over-expression of Atg1 fragments that contained the TID led to diminished levels of Atg1 kinase and autophagy activities. Autophagy levels were assessed in rapamycin-treated cell extracts, which contained a genomic copy of Atg1 and the indicated Atg1 fragments. Atg1 kinase activity was also interfered by the overexpression of the indicated Atg1 fragments. The levels of Thr-226 phosphorylation were used to examine Atg1 kinase activity. (CTerm, 331 – 897 fragment of Atg1; 550 – C, 550 – 897 fragment of Atg1; 600 – C, 600 – 897 fragment of Atg1; 731 – C, 731 – 897 fragment of Atg1.)
Figure 4.2 Atg13 and Atg17 played a distinct role in regulating the Atg1 kinase and the autophagy activities.
Figure 4.3 Atg1 self-association domain is within the Atg1 kinase domain.

(A) The scheme shows the kinase domain (KIN) indicated as “KINASE” and the position of amino Figure 4.3. Atg1 self-association domain is within the Atg1 kinase domain. acid residue at the Atg1 protein. (B) Examining the domain of the Atg1 self-association. An HA-tagged full-length Atg1 was precipitated from cell extracts and the relative amounts of the indicated myc-tagged Atg1 fragments associated were assessed by Western blotting. The bottom panel showed the total amount of Atg1 proteins present in cells measured by Western blotting. (C) The strength of binding between the full-length and the kinase domain of Atg1 protein to the full-length Atg1 protein. The lane 2 and 4, show 20 and 50-times dilution of the original precipitation used for the full-length Atg1. (FL, the full-length Atg1; KIN, the kinase domain of Atg1; CTerm, 331 – 897 fragment of Atg1; Bullet, degradation bands.)
Figure 4.4 Atg13 was required for the Atg1 self-interaction.

(A) The Atg1 self-association was assessed in *atg13Δ* and *atg17Δ* cells with two differently tagged full-length Atg1 as described in Figure 4.3 B. (B) Overexpression of Atg13 enhanced Atg1 self-association. Atg13 and Atg17 were overexpressed in the cells and Atg1 self-interaction was examined.
Figure 4.5 Overexpression of Atg1 fragments contained the TID domain interfered Atg1 self-interaction.

(A) Over-expression of the 331-897 fragment of Atg1 (CTerm) disrupted the Atg1 self-association. The indicated fragments of Atg1 were expressed in cells containing two differently tagged versions of the full-length Atg1, in which Atg1 self-association was assayed. (B) The Atg1-Atg1 interaction was disrupted in a dose-dependent manner by the addition of increasing amounts of the CTerm fragment of Atg1. In this experiment, two different cell extracts were mixed, one that contained both the HA- and myc-tagged full-length versions of Atg1 and a second that contained the CTerm domain. Increasing amounts of the second extract containing the CTerm fragment were added to a constant amount of the first extract. The relative level of Atg1 self-association was analyzed. (C) The relative ability of different Atg1 fragments to disrupt the Atg1 self-association was assessed in an extract mixing experiment similar to that described above in (B). In these experiments, the second extracts contained the indicated fragments of Atg1 (600 – C, 650 – C, 731 – C). The first extracts again contained both the HA- and myc-tagged full-length Atg1 proteins. The effect of adding the extract only is shown in the vector control lanes (Vec). (mAtg1, myc-tagged Atg1; hAtg1, HA-tagged Atg1; KIN, the kinase domain of Atg1; CTerm, the 331–897 fragment of Atg1; 600 – C, the 600–897 fragment of Atg1; 650 – C, the 650–897 fragment of Atg1; 731 – C, the 731–897 fragment of Atg1.)
Figure 4.5 Overexpression of Atg1 fragments contained the TID domain interfered Atg1 self-interaction.
Figure 4.6 Atg1$^{T226E}$ variant was self-associated in the Atg13 independent fashion.

(A) The phosphomimetic Atg1 at Thr-226 bypassed the requirement of Atg13 for the Atg1 self-association. The indicated Atg1 variants were co-expressed in two different tagged versions in cells, in which the Atg1 self-interaction assays were performed. (B) A model proposing that Atg13 (shown as a dimer) promotes the autophosphorylation at T226 within the Atg1 activation loop and the Atg1 self-association. See text for additional details. (WT, the wild type Atg1; A, Atg1$^{T226A}$ variant; E, Atg1$^{T226E}$ variant.)
Chapter 5

Synopsis

Since the discovery of autophagy four decades ago, progress has been made to understand this cellular process during which a double-membrane vesicle, the autophagosome, delivers cytoplasmic material to lysosomes for degradation and recycling. Insights into the molecular control of autophagy were largely acquired in *Saccharomyces cerevisiae*, including identification of *ATG* genes, requirement of autophagosome assembly and the first link of regulatory control to the signaling transduction pathway. Knowledge and research tools developed from yeast have shaped the framework for further studies in higher eukaryotes by virtue of conservation of the autophagy process. However, research has centered more on structural aspects of the Atg proteins and the molecular mechanism of autophagy while less has been discovered regarding the regulation of autophagy. The complexity of autophagy regulation in eukaryotes was becoming apparent from recent molecular analyses in mammals \(^97-101\). Our lab and others have shown that the Tor and PKA pathways are critical regulators of autophagy through targeting the Atg1 protein complex. Tor and PKA interfere with the cellular localization of Atg1 and Atg13 in the non-starved condition and Tor inhibits Atg1 kinase activity by preventing Atg1-Atg13 interaction.
The study reported here extended these observations and investigated the regulation of Atg1 in the induction of autophagy. We identified a specific phosphorylation event on Atg1 as a potential regulatory point in the autophagy pathway in *Saccharomyces cerevisiae*. This phosphorylation occurs within the Atg1 activation loop at a threonine residue, Thr-226 that is conserved in all Atg1 orthologs. Replacing this threonine with a nonphosphorylatable residue resulted in a loss of Atg1 protein kinase activity and a failure to induce autophagy. Substitution of this threonine for a phosphomimetic residue, Glu or Asp led to constitutively active Atg1 kinase activity, but failed in the induction of autophagy. This phosphorylation required two known regulators of Atg1 activity, Atg13 and Atg17. In addition, T226 phosphorylation was associated with an autophosphorylated form of Atg1 that was found specifically in cells undergoing the autophagy process. Overall the data suggested that autophosphorylation within the Atg1 activation loop may represent a point of regulatory control for this degradative process.

We furthered our studies in the structural analyses of Atg1 and successfully mapped the Atg1 self-association domain and the Atg1-Atg13 interaction domain (TID) of Atg1 at N- and C-terminal region of Atg1 respectively. The collective data indicate that promoting the activation loop phosphorylation within Atg1 is the primary role for two key conserved regulators of Atg1 activity, Atg13 and Atg17. Atg13 interacts with Atg1 specifically to stimulate this T226 phosphorylation and promote an Atg1-Atg1 self-association. Our analysis also suggested that although Atg17 is required for Atg1 kinase activity, however the functional role of Atg17 in activation of Atg1 is distinct from Atg13. The discovery of the role of Atg13 provides important insight into the mechanistic
role of Atg13 in the induction of the Atg1 kinase activity and hence the autophagy process.

To extend our understanding of the control of Atg1 by phosphorylation, we used multiple means to identify and characterize additional sites of phosphorylation in the yeast Atg1. One site, Ser-34 was of special interest as it was found to be in a highly conserved G-loop motif within the Atg1 kinase domain. We showed here that this site served an inhibitory function in Atg1 in vivo. Further work is needed to determine the molecular mechanism of this inhibitory modification of Atg1 and to identify the protein kinase responsible for Ser-34 phosphorylation. Additionally, we identified Ser-390 as the site of autophosphorylation responsible for the anomalous migration observed for Atg1 on SDS-polyacrylamide gels. This slow-migrating hyperphosphorylated band is specifically associated with the activated Atg1 kinase activity isolated from the starved condition, which we developed this into an effective assay to monitor in vivo Atg1 kinase activity. The analyses identified a number of potential sites of regulation in the Atg1 protein that will hopefully serve as a framework for future work with this enzyme.

This body of work demonstrates that the Atg1 kinase complex is an important regulatory point in autophagy and provides new insight into the regulation of Atg1 protein kinase. Furthermore, this study identified multiple phosphorylation sites and also contributed to the better understanding of activation of the Atg1 kinase activity during the induction of autophagy. However, further work is needed to resolve many unanswered questions that this study cannot cover. First, it is intriguing that the phosphomimetic Atg1 at Thr-226 fails to induce autophagy. One of many possibilities is that this Atg1 variant is unable to recognize the downstream substrates, which are important for conducting
autophagy function. Indeed, this constitutively active Atg1 variant failed to phosphorylate an exogenous substrate, myelin basic protein, used because there is no bona fide substrate for Atg1. Second, the precise function of Atg17 in promoting Atg1 kinase activity needs to be revealed. Besides Atg17 acts as a scaffold protein to organize Atg proteins to the PAS, little is known about the function of Atg17. Although we showed Atg17 is required for activation of Atg1, our data also suggested that perhaps Atg17 also works as a negative feedback control for Atg1 protein kinase since overexpression of Atg17 inhibits both Atg1 kinase and autophagy activities. Last but not the least, identification of authentic substrates for Atg1 will be crucial for the ultimate understanding of the regulation of autophagy. We have identified the first autophosphorylation site at Ser-390 of Atg1 besides activation loop phosphorylation, which may provide us with a hint about Atg1 substrate consensus site. Based on the neighboring sequence of Ser-390, we can conduct protein database searches or design a specific Atg1 protein chip with fabricated peptide arrays to identify potential Atg1 substrates.

To summarize, data presented here have uncovered features of the regulation of Atg1 kinase activity in autophagy and emphasized the significance of Atg1 protein complex in control of autophagy. On account of there being a conserved Atg1 protein complex among all eukaryotes, the findings in this study provide useful regulatory insights into Atg1 in multicellular eukaryotes. Further investigation of the Atg1 protein complex will shed the light on the molecular mechanism of autophagy with an ultimate goal to benefit human health.


50. Cheong, H., Nair, U., Geng, J. & Klionsky, D.J. The Atg1 kinase complex is involved in the regulation of protein recruitment to initiate sequestering vesicle


Appendix A: MS/MS spectra for all the identified phosphopeptides
Figure A1. MS/MS spectra for each of the identified phosphorylation sites in Atg1 – Ser-34. Peptide 29-40, [M + 3H]^{3+} precursor ion at m/z 469.9. The amino acid sequences and the respective b and y ions produced are shown in each spectrum, the phosphorylated serine or threonine residues are indicated with “p”. The product ions corresponding to neutral loss of H_{2}O are indicated with circles (O). Asterisks indicate product ions derived from neutral loss of NH_{3}. The b/y-98 indicates product ions carrying a neutral loss of H_{3}PO_{4} (98 Da).
Figure A2 MS/MS spectra for each of the identified phosphorylation sites in Atg1 - Thr-129. Peptide 119-133, [M + 3H]^{3+} precursor ion at m/z 651.3. The amino acid sequences and the respective b and y ions produced are shown in each spectrum, the phosphorylated serine or threonine residues are indicated with “p”. The product ions corresponding to neutral loss of H_{2}O are indicated with circles (O). Asterisks indicate product ions derived from neutral loss of NH_{3}. The b/y-98 indicates product ions carrying a neutral loss of H_{3}PO_{4} (98 Da).
Figure A3. MS/MS spectra for each of the identified phosphorylation sites in Atg1 - Ser-304. Peptide 300-313, [M + 2H]^{2+} precursor ion at m/z 871.9. The amino acid sequences and the respective b and y ions produced are shown in each spectrum, the phosphorylated serine or threonine residues are indicated with “p”. The product ions corresponding to neutral loss of H$_2$O are indicated with circles (O). Asterisks indicate product ions derived from neutral loss of NH$_3$. The b/y-98 indicates product ions carrying a neutral loss of H$_3$PO$_4$ (98 Da).
Figure A4. MS/MS spectra for each of the identified phosphorylation sites in Atg1 - Ser-365. Peptide 362-388, [M + 4H]$^{4+}$ precursor ion at $m/z$ 729.9. The amino acid sequences and the respective b and y ions produced are shown in each spectrum, the phosphorylated serine or threonine residues are indicated with “p”. The product ions corresponding to neutral loss of H$_2$O are indicated with circles (O). Asterisks indicate product ions derived from neutral loss of NH$_3$. The b/y-98 indicates product ions carrying a neutral loss of H$_3$PO$_4$ (98 Da).
Figure A5. MS/MS spectra for each of the identified phosphorylation sites in Atg1 - Ser-515, previously discovered as a PKA site. Peptide 513-527; \([M + 3H]^{3+}\) precursor ion at \(m/z\) 565.6. The amino acid sequences and the respective \(b\) and \(y\) ions produced are shown in each spectrum, the phosphorylated serine or threonine residues are indicated with “\(p\)”. The product ions corresponding to neutral loss of H\(_2\)O are indicated with circles (\(\circ\)). Asterisks indicate product ions derived from neutral loss of NH\(_3\). The \(b/y-98\) indicates product ions carrying a neutral loss of H\(_3\)PO\(_4\) (98 Da).
Figure A6. MS/MS spectra for each of the identified phosphorylation sites in Atg1 - Ser-533. Peptide 528-535, [M + 2H]^{2+} precursor ion at m/z 434.7. The amino acid sequences and the respective b and y ions produced are shown in each spectrum, the phosphorylated serine or threonine residues are indicated with “p”. The product ions corresponding to neutral loss of H_{2}O are indicated with circles (O). Asterisks indicate product ions derived from neutral loss of NH_{3}. The b/y-98 indicates product ions carrying a neutral loss of H_{3}PO_{4} (98 Da).
Figure A7. MS/MS spectra for each of the identified phosphorylation sites in Atg1 - Ser-551/552. Peptide 536-575, [M + 4H]^{4+} precursor ion at m/z 1163.4. The amino acid sequences and the respective b and y ions produced are shown in each spectrum, the phosphorylated serine or threonine residues are indicated with “p”. The product ions corresponding to neutral loss of H\_2O are indicated with circles (\textsuperscript{0}). Asterisks indicate product ions derived from neutral loss of NH\_3. The b/y-98 indicates product ions carrying a neutral loss of H\_3PO\_4 (98 Da).
Figure A8. MS/MS spectra for each of the identified phosphorylation sites in Atg1 - Thr-590. Peptide 587-602, [M + 2H]^{2+} precursor ion at m/z 890.9. The amino acid sequences and the respective b and y ions produced are shown in each spectrum, the phosphorylated serine or threonine residues are indicated with “p”. The product ions corresponding to neutral loss of H$_2$O are indicated with circles ('). Asterisks indicate product ions derived from neutral loss of NH$_3$. The b/y-98 indicates product ions carrying a neutral loss of H$_3$PO$_4$ (98 Da).
Figure A9. MS/MS spectra for each of the identified phosphorylation sites in Atg1 - Ser-621. Peptide 614-625, [M + 2H]^{2+} precursor ion at m/z 707.3. The amino acid sequences and the respective b and y ions produced are shown in each spectrum, the phosphorylated serine or threonine residues are indicated with “p”. The product ions corresponding to neutral loss of H_{2}O are indicated with circles (O). Asterisks indicate product ions derived from neutral loss of NH_{3}. The b/y-98 indicates product ions carrying a neutral loss of H_{3}PO_{4} (98 Da).
Figure A10. MS/MS spectra for each of the identified phosphorylation sites in Atg1 - Ser-677. Peptide 675-688, [M + 2H]^{2+} precursor ion at m/z 774.8. The amino acid sequences and the respective b and y ions produced are shown in each spectrum, the phosphorylated serine or threonine residues are indicated with “p”. The product ions corresponding to neutral loss of H_2O are indicated with circles (O). Asterisks indicate product ions derived from neutral loss of NH_3. The b/y-98 indicates product ions carrying a neutral loss of H_3PO_4 (98 Da).
Figure A11. MS/MS spectra for each of the identified phosphorylation sites in Atg1 - Ser-680. Peptide 675-688, [M + 2H]^{2+} precursor ion at m/z 774.8. The amino acid sequences and the respective b and y ions produced are shown in each spectrum, the phosphorylated serine or threonine residues are indicated with “p”. The product ions corresponding to neutral loss of H\textsubscript{2}O are indicated with circles (\(\circ\)). Asterisks indicate product ions derived from neutral loss of NH\textsubscript{3}. The b/y-98 indicates product ions carrying a neutral loss of H\textsubscript{3}PO\textsubscript{4} (98 Da).
Figure A12. MS/MS spectra for each of the identified phosphorylation sites in Atg1 - Ser-683. Peptide 675-688, [M + 2H]^{2+} precursor ion at m/z 774.8. The amino acid sequences and the respective b and y ions produced are shown in each spectrum, the phosphorylated serine or threonine residues are indicated with “p”. The product ions corresponding to neutral loss of H₂O are indicated with circles ('). Asterisks indicate product ions derived from neutral loss of NH₃. The b/y-98 indicates product ions carrying a neutral loss of H₃PO₄ (98 Da).
Figure A13. MS/MS spectra for each of the identified phosphorylation sites in Atg1 - Ser-769. Peptide 765-780, [M + 3H]^{3+} precursor ion at m/z 647.3. The amino acid sequences and the respective b and y ions produced are shown in each spectrum, the phosphorylated serine or threonine residues are indicated with “p”. The product ions corresponding to neutral loss of H$_2$O are indicated with circles (O). Asterisks indicate product ions derived from neutral loss of NH$_3$. The b/y-98 indicates product ions carrying a neutral loss of H$_3$PO$_4$ (98 Da).
Figure A14. MS/MS spectra for each of the identified phosphorylation sites in Atg1 - Ser-783. Peptide 781-791, [M + 2H]^{2+} precursor ion at m/z 659.3. The amino acid sequences and the respective b and y ions produced are shown in each spectrum, the phosphorylated serine or threonine residues are indicated with “p”. The product ions corresponding to neutral loss of H\textsubscript{2}O are indicated with circles (O). Asterisks indicate product ions derived from neutral loss of NH\textsubscript{3}. The b/y-98 indicates product ions carrying a neutral loss of H\textsubscript{3}PO\textsubscript{4} (98 Da).