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Biochemical and Structural Characterization of the Atg8/LC3 Lipidation Pathway

Abstract

Atg8 and its eukaryotic orthologues LC3 and GABARAP family proteins (referred here to Atg8 family proteins) play crucial roles in autophagy through their covalent ligation to lipids, typically phosphatidylethanolamine (PE), in a process known as lipidation. Lipidation of Atg8 family proteins regulates numerous facets of the autophagy process, including regulating expansion of the phagophore membrane, recruiting selected cargoes for degradation, and providing an autophagosome membrane-bound platform mediating dynamic interactions with other regulatory proteins. Atg8 family proteins are ubiquitin-like proteins (UBLs), and their lipidation involves a divergent UBL conjugation cascade including Atg7, Atg3, and Atg12–Atg5–Atg16 acting as E1, E2, and E3 enzymes, respectively. Atg7 initiates Atg8 conjugation by catalyzing their C-terminal adenylation and conjugation to the catalytic cysteine of Atg3. Ultimately, the Atg12–Atg5–Atg16 complex catalyzes Atg8 ligation to a primary amino group on PE or other acceptor lipids. Molecular mechanisms underlying Atg8 lipidation remain poorly understood despite association of Atg3, the E1 Atg7, and the composite E3 Atg12–Atg5–Atg16 with pathologies including cancers, infections, and neurodegeneration. The first part of this dissertation work describes methods for expressing and purifying human LC3 or GABARAP, ATG7, ATG3, and the ATG12–ATG5–ATG16L1 complex for in vitro studies of LC3/GABARAP lipidation; based on these protocol established, we report that an Atg3 element we term E123IR (E1, E2, and E3-interacting region) is an allosteric switch, by studying yeast enzymes. Nuclear magnetic resonance (NMR), biochemical, crystallographic and genetic data collectively indicate that in the absence of the enzymatic cascade, the Atg3E123IR makes intramolecular interactions restraining Atg3's catalytic loop, while E1 and E3 enzymes directly remove this brace to conformationally activate Atg3 and elicit Atg8 lipidation in vitro and in vivo. We propose that Atg3's E123IR protects the E2~UBL thioester bond from wayward reactivity toward errant nucleophiles, while Atg8 lipidation cascades

Document Type

Dissertation

Degree Name

Doctor of Philosophy (PhD)

Program

Biomedical Sciences

Research Advisor

Brenda A. Schulman, PhD

Keywords

Atg8, autophagy, E2, ligase, lipidation, ubiquitin-like protein

Subject Categories

Medical Sciences | Medicine and Health Sciences

UNIVERSITY OF TENNESSEE HEALTH SCIENCE CENTER

DOCTOR OF PHILOSOPHY DISSERTATION

**Biochemical and Structural Characterization of
the Atg8/LC3 Lipidation Pathway**

Author:
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Advisor:
Brenda A. Schulman, PhD

*A Dissertation Presented for The Graduate Studies Council of
The University of Tennessee Health Science Center
in Partial Fulfillment of the Requirements for the
Doctor of Philosophy degree from
The University of Tennessee*

in

*Biomedical Sciences: Microbiology, Immunology, and Biochemistry
College of Graduate Health Sciences*

May 2020

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DEDICATION

I dedicate my dissertation to my parents Guoqing Zheng and Wei Liu, my loving grandparents, and my incredible husband Dr. Xi-Zhi (Johnny) Guo. I am deeply indebted to their unconditional and endless love, care and supports that have guarded me through not only this rocky journey pursuing a doctoral degree in a foreign country, but also through my entire life.

I dedicate this work to my son Elliott Guo, who has been a surprising gift and the greatest bless. He has empowered me to always stay calm, strong-minded and optimistic facing up unexpected difficulties in science and personal life.

I dedicate this work to my PhD advisor Dr. Brenda A. Schulman for being the best mentor possible. I am grateful to her tremendous patience and efforts guiding me into the mansion of science, as well as to her passionate and generous personalities that inspire me to always keep my decency both in science and in personal life.

I also dedicate this work to my sweet cat Earl, my affectionate cousin Dr. Chen Xu, my childhood friend Wan-Ting Dong, and my closest colleague Dr. Yu Qiu. I sincerely appreciate their constant supports and warm companionship.

ACKNOWLEDGEMENTS

I would like to thank the rest of my dissertation committee members Dr. Charles Rock, Dr. Eric Enemark, Dr. Mondira Kundu and Dr. Julio Cordero-Morales for their great mentorship and invaluable advice. I am grateful to all (previous) Schulman lab members, especially Dr. Danny Scott, Dr. Kuen-Phon Wu, Dr. Nicolas Brown, Dr. Ryan VanderLinden, Dr. Randy Watson, Shein Ei Cho, Shelia Bozeman and David Miller, for their generous helps inside and outside the lab. I am thankful to Dr. Xu Liu and Dr. Daniel Klionsky at University of Michigan, for their crucial experimental data and remarks that composes an essential part of my final dissertation. I am appreciative of Dr. Grace Royappa and Dr. Darcie Miller for their professional technical facilitations. I show gratitude for Dr. Ryan Potts and his lab members for their scientific and mental supports. I thank my friends Dr. Yinan Gong, Dr. Qifan Zhu, Dr. Qianxia (Sherry) Zhang, Dr. Cheng Tian, Qiyue (Ceci) Chen, Xin Lan and Hengyu Jiang, and Thomas lab members for their companionship.

ABSTRACT

Atg8 and its eukaryotic orthologues LC3 and GABARAP family proteins (referred here to Atg8 family proteins) play crucial roles in autophagy through their covalent ligation to lipids, typically phosphatidylethanolamine (PE), in a process known as lipidation. Lipidation of Atg8 family proteins regulates numerous facets of the autophagy process, including regulating expansion of the phagophore membrane, recruiting selected cargoes for degradation, and providing an autophagosome membrane-bound platform mediating dynamic interactions with other regulatory proteins. Atg8 family proteins are ubiquitin-like proteins (UBLs), and their lipidation involves a divergent UBL conjugation cascade including Atg7, Atg3, and Atg12–Atg5–Atg16 acting as E1, E2, and E3 enzymes, respectively. Atg7 initiates Atg8 conjugation by catalyzing their C-terminal adenylation and conjugation to the catalytic cysteine of Atg3. Ultimately, the Atg12–Atg5–Atg16 complex catalyzes Atg8 ligation to a primary amino group on PE or other acceptor lipids.

Molecular mechanisms underlying Atg8 lipidation remain poorly understood despite association of Atg3, the E1 Atg7, and the composite E3 Atg12–Atg5–Atg16 with pathologies including cancers, infections, and neurodegeneration. The first part of this dissertation work describes methods for expressing and purifying human LC3 or GABARAP, ATG7, ATG3, and the ATG12–ATG5–ATG16L1 complex for *in vitro* studies of LC3/GABARAP lipidation; based on these protocol established, we report that an Atg3 element we term E123IR (E1, E2, and E3-interacting region) is an allosteric switch, by studying yeast enzymes. Nuclear magnetic resonance (NMR), biochemical, crystallographic and genetic data collectively indicate that in the absence of the enzymatic cascade, the Atg3^{E123IR} makes intramolecular interactions restraining Atg3's catalytic loop, while E1 and E3 enzymes directly remove this brace to conformationally activate Atg3 and elicit Atg8 lipidation *in vitro* and *in vivo*. We propose that Atg3's E123IR protects the E2~UBL thioester bond from wayward reactivity toward errant nucleophiles, while Atg8 lipidation cascade enzymes induce E2 active site remodeling through an unprecedented mechanism to drive autophagy.

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LIST OF ABBREVIATIONS

AD	Adenylation domain
ATG	Autophagy related gene
BME	Beta-mercaptoethanol
CSP	Chemical shift perturbation
CTD	C-terminal domain
DTT	Dithiothreitol
E123IR	E1, E2 and E3 interactive region
ER	Endoplasmic reticulum
FPLC	Fast protein liquid chromatography
FR	Flexible region
GABARAP	Gamma-aminobutyric acid receptor-associated protein
GST	Glutathione S-transferase
HECT	Homologous to the E6-AP Carboxyl Terminus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HR	Handle region
LC3B	Microtubule-associated proteins 1A/1B light chain 3B
MES	2-(N-morpholino)ethanesulfonic acid
NMR	Nuclear magnet resonance
NTD	N-terminal domain
PAGE	Polyacrylamide
PEG	Polyethylene glycol
RBR	RING between RING
RING	Really interesting gene
RNF4	RING finger protein 4
SDS	Sodium dodecyl sulfide
SNARE	N-ethylmaleimide-sensitive factor attachment protein receptor
STDEV	Standard deviation
SUMO	Small ubiquitin-like modifier
TEV	Tobacco Etch Virus nuclear-inclusion-a endopeptidase

TRIS	trisaminomethane
UBE2D1	Ubiquitin-conjugation enzyme E2 D1
UBL	Ubiquitin-like protein
UDF	Ubiquitin-fold domain
ULK	Unc-51-like kinase

CHAPTER 1. INTRODUCTION

Backgrounds

Autophagy

Autophagy is a major catabolic pathway by which eukaryotic cells degrade and recycle diverse cellular materials (Yang and Klionsky, 2010). Sectors of the cytosol are first sequestered within a double-membrane-bound autophagosome, and then degraded upon autophagosome fusion with the lysosome in higher eukaryotes or vacuole in yeast (Nakatogawa et al., 2009). Cargoes directed to autophagosomes for degradation include protein assemblies, organelles, misfolded macromolecules, debris, and pathogenic bacteria (Galluzzi et al., 2017; Gatica et al., 2018; Stolz et al., 2014) (**Figure 1-1**). Autophagy plays important roles in cellular responses upon various forms of stress including starvation, toxicities and pathogenic infections. It is an indispensable cell activity in context of homeostasis, cellular differentiation and development. Dysregulation of autophagy is associated with metabolic, neurologic, and oncologic disorders (Amaravadi et al., 2016; Dikic and Elazar, 2018; Levy et al., 2017; Mizushima et al., 2008) and human diseases, including cancer, Parkinson's disease, Crohn's disease, liposome storage disorders, and so forth (van Beek et al., 2018).

The process of autophagy

Autophagy is a tightly regulated multi-stage activity, which can be dissected into 6 steps: initiation, phagophore nucleation, phagophore membrane expansion and cargo engulfment, autophagosome fusion with the vacuole or lysosome, cargo degradation, and recycling of the processed materials (Mizushima, 2007). Triggered by a wide range of signaling pathways, autophagy is initiated by activation of the unc-51-like kinase (ULK) complex. This step sequentially activates the phosphoinositide 3-kinase (PI3K) complex and Atg9 which collectively mediate the phagophore nucleation (Lane et al., 2017). Once the nucleation is completed, membrane from multiple potential sources, for example mitochondria (Lyamzaev et al., 2018; Rambold and Lippincott-Schwartz, 2011), endosomes (Longatti et al., 2012; Puri et al., 2013), endoplasmic reticulum (ER) (Carlos Martín Zoppino et al., 2010), Golgi bodies (Bodemann et al., 2011; van der Vaart et al., 2010), and even plasma membrane (Ravikumar et al., 2010), gathers at the phagophore as forming a double membrane organelle called autophagosome (Carlsson and Simonsen, 2015). This process is followed by other autophagic machinery being recruited to the isolated membrane preparing for the membrane elongation. The key proteins include WD-repeat protein interacting with phospho-inositides (WIPI) proteins (Proikas-Cezanne et al., 2015) and the enzyme cascade required for Atg8 family protein lipidation (Rubinsztein et al., 2012). Atg8 family is a distinct subgroup of the ubiquitin-like (UBL) proteins. They modify phagophore membrane lipids through a chemical reaction similarly to other

Figure 1-1. The process of autophagy.

After Atg8/ LC3 family proteins being ligated to the phosphatidylethanolamine(PE) on phagophore membrane is followed by membrane elongation and engulfment, meanwhile autophagic cargos are recruited to the phagophore via Atg8 binding with a variety of specific adaptor proteins that recognize and interact with the cargos, forming a the double membrane vesicle termed as autophagosome. Autophagosome fuses with lysosome (or vacuole in yeast) and utilizes the lysosomal hydrolases to degrade the cargos for re-utilization.

ubiquitin-like protein ligations, featured by the glycine at the truncated C-terminus of Atg8 family proteins being covalently ligated to amino group of phosphatidylethanolamine (PE) (Nakatogawa et al., 2007). Membrane-linked Atg8 family proteins not only participate in the phagophore elongation and fusion, but also promote autophagosome closure (Feng et al., 2014; Fujita et al., 2008a). Moreover, autophagosome-coated Atg8 family proteins are responsible for autophagic receptor recognition and recruitment. This activity is realized by a specific interaction, where a hydrophobic groove on Atg8 captures unique peptide sequences termed as the Atg8 interactive motif (AIM, in yeast or fungi) or LC3 interactive region (LIR, in higher organisms) harbored by autophagic adaptors or other autophagy related binding partners (Birgisdottir et al., 2013). As phagophore membrane expands and cargo engulfment proceeds, autophagosome eventually seals, then most of Atg8 molecules are cleaved from the outer-membrane by Atg4. In the next step, autophagosome first migrates with facilitation from the microtubule system, then fuse with vacuole or lysosome under the regulation of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE), Rab family proteins, PI3K complex and Rubicon (Hikita et al., 2018). The inner membrane and the engulfed cargos of the autophagosome are therefore degraded by vacuolar or lysosomal hydrolases. Finally, the resulting metabolites are exported to the cytosol for reutilization.

Atg8 family protein lipidation and autophagy

Autophagy-related 8 protein (Atg8) family is conserved across eukaryotic species. Atg8 is the only family member found in yeast and other fungus, whereas other three subfamilies: microtubule-associated protein 1 light chain 3 (MAP1LC3 or LC3), γ -aminobutyric acid receptor-associated protein (GABARAP) and Golgi-associated ATPase enhancer of 16 kDa (GATE-16) exist in higher organisms including animals and plants. Despite residue variation in several positions across the whole proteins, Atg8 family members share major similarity in amino acid sequences and protein structures (**Figure 1-2**). Structural studies in different Atg8 family proteins, including, but not limited to, Atg8s from multiple yeast organisms and the human LC3 and GABARAP family members, have revealed significant conformational similarity to ubiquitin. However, comparing to other UBL family members, in addition to the globular ubiquitin domain, Atg8 family proteins feature an additional N-terminal tail with moderate flexibility which consists of two helices connected by a short linker (**Figure 1-2**).

As described in 1.1.2, many facets of autophagy rely on Atg8 in yeast (or six LC3- and GABARAP-family ubiquitin-like proteins in higher eukaryotes, here referred collectively to Atg8 family members) and the E1-E2-E3 enzymatic cascade that links Atg8's C-terminus to the primary amine group of phosphatidylethanolamine (PE) lipid molecules (Dikic, 2017; Ichimura et al., 2000; Shpilka et al., 2011, 2012). As initiation of the lipidation process, the C-terminal tail of Atg8 family protein is cleaved by Atg4, exposing a glycine residue at the very end accessible by nucleophiles. Under tight regulation of autophagic machinery, this truncated version of Atg8 forms a thioester bond-linked complex with Atg7 (E1) via sidechains of C-terminal glycine and Atg7

Figure 1-2. Structure comparison of Atg8 and LC3 to other ubiquitin like proteins.

In addition to the globular domain that shared by all ubiquitin-like proteins, Atg8/LC3 family proteins are featured by a N-terminal helical extension.

catalytic cysteine. Ultimately, lipidated Atg8 family members serve as bridges between membranes and the cytosol, where their ubiquitin-like fold domains recruit AIM or LIR sequences in a staggering array of partner proteins involved in various aspects of autophagy (Birgisdottir et al., 2013; Noda et al., 2010; Rogov et al., 2014; Wild et al., 2014). These include regulators and effectors of autophagosome biogenesis that promote membrane tethering and growth of the autophagosome, as well as adaptor proteins that in turn recruit cargoes for degradation (Zaffagnini and Martens, 2016). Accordingly, Atg8 and its conjugation system enzymes are essential for autophagy in yeasts, and autophagy is severely impaired in their absence in mammalian cells (Tsuboyama et al., 2016). Lipidated Atg8-family members have also been implicated in protein recruitment to membranes in processes that are unrelated to autophagy in mammalian cells. Therefore, given these important roles of Atg8 and its conjugation system enzymes, it is important to understand molecular mechanisms regulating Atg8 lipidation.

The process of Atg8 lipidation

The process of Atg8 lipidation largely follows the similar procedure of other ubiquitin-like protein ligation system, whereas the ubiquitin or ubiquitin-like protein is delivered through a three enzyme (E1, E2, and E3) cascade before being conjugated to the final substrate (Ohsumi and Mizushima, 2004). The UBL, or Atg8 processed by Atg4 in this context, first uses its C-terminal glycine to generate a thioester bond with the catalytic cysteine in E1 Atg7 (Tanida et al., 1999); this E1-UBL complex is disassociated upon interacting with E2 Atg3, where UBL is transferred to E2 and forms a new thioester bond with the catalytic cysteine of E2 (Tanida et al., 2002); finally, facilitated by E3 Atg12–Atg5–Atg16, UBL is discharged from E2 and ligated to the target amine group located within the substrate PE (Hanada et al., 2007; Walczak and Martens, 2013) (**Figure 1-3**). While many UBL family members are able to coordinate with diverse E2s and E3s, and target an even wider range of substrates, Atg8, however, stays strictly specific to E2-like protein Atg3, E3-like protein complex Atg12–Atg5–Atg16 and its only lipid substrate - phosphatidylethanolamine (PE); Atg8 is also known as the only UBL that modifies amine group in lipid substrates other than lysines in protein substrates.

Atg7, the E1 for Atg8, harbors a N-terminal domain (NTD) and a C-terminal domain (CTD) (Taherbhoy et al., 2011). Atg7 adenylation domain (AD), which is a subdomain of the CTD, mediates self-dimerization as ADs in the canonical E1, and is both structurally and functionally comparable to the canonical E1 AD dimers (Taherbhoy et al., 2011). The flat dimeric interface serves as a platform to interact with the UBL as in other E1s. However, lacking the E1's signature ubiquitin-fold domain (UFD) but replacing it by the unique NTD, the overall structure of Atg7 is distinct from other E1s. Unlike canonical E1 using UFD to recruit E2, Atg7, as dimerized via AD, interacts with Atg3 core domain by one copy of NTD *in cis*, and meanwhile binds an exclusive motif in Atg3 that is absent in other canonical E2s by the other copy *in trans* (Taherbhoy et al., 2011).

Figure 1-3. The process of Atg8 lipidation.

The lipidation of Atg8/LC3 family protein utilizes E1-E2-E3 cascade similar to other ubiquitin-like protein conjugation system. Atg8 first forms a thioester intermediate with activated E1 Atg7; the thioester bond is nucleophilically attacked by Atg3 catalytic cysteine, resulting in breaking down of the Atg7-Atg8 bond and formation of a new thioester bond between Atg8 with E2 Atg3 catalytic cysteine; in the final step, E3 Atg12-Atg5-Atg16 facilitates Atg8 being discharged from Atg3 and ligated to PE.

The unique E2 for Atg8, Atg3, features a core domain that resembles other canonical E2 folds and harbors an E2 catalytic center including a topologically conserved catalytic cysteine (Yamada et al., 2007). In addition, it contains a 20-amino acid N-terminal helical extension (N-term) with the ability of sensing membrane curvature (Nath et al., 2014), an approximately 100-amino acid unstructured insertion in the middle of the core domain termed as the flexible region (FR) (Yamada et al., 2007), and an unique handle region (HR) downstream of the catalytic cysteine (Yamada et al., 2007). Upon activation by the E3-like enzyme Atg12–Atg5–Atg16 complex, the Atg3 catalytic center undergoes conformational changes, during which the catalytic cysteine and its surrounding regulative residues are rearranged to positions favored by Atg8-ligation (Sakoh-Nakatogawa et al., 2013). However, the functions of the FR and HR in Atg8 lipidation remain obscure, though human protein study suggested that a short motif within the FR adopts a helical folding upon binding to the E3 compartment ATG12 (Metlagel et al., 2013). And such interaction is indispensable for LC3 conjugation activity. Due to lack of structural evidences, it is also unrevealed about the full conformational requirements to activate the Atg3~Atg8 thioester intermediate as well as the full picture of Atg3–Atg12–Atg5–Atg16 interaction.

The E3-like enzyme Atg12–Atg5–Atg16 resembles the ubiquitin-like protein ligation process. As a ubiquitin-like protein itself, Atg12 is conjugated to the substrate Atg5 via an isopeptide bond, catalyzed by its E1 Atg7 and E2 Atg10 without involvement of an E3 (Hanada and Ohsumi, 2005; Ohsumi and Mizushima, 2004). Atg16 later joins the Atg12–Atg5 complex as a non-covalent yet stable binding partner. Both human ATG5 and ATG16L were revealed to have intrinsic binding abilities to lipids (Lystad et al., 2019; Romanov et al., 2012). Taken together with the membrane curvature sensing function of the ATG3 N-terminal helix (Nath et al., 2014), this may indicate a potential recruiting mechanism of the Atg3~Atg8 intermediate to the phagophore membrane. Neither the Atg12–Atg5–Atg16 E3 complex nor its individual compartments shows conservation in sequence to any of the three families of E3 (the RING family, the HECT family, and the RBR family); studies in multiple organisms including human and yeast also reveal no structural similarity between Atg12–Atg5–Atg16 and other E3 ligases. With these significant differences between autophagy and canonical E1, E2, and E3s, it is challenging to study the mechanism of Atg8 lipidation based on existing UBL conjugation models. On the other hand, all these distinctive features of the Atg8 lipidation cascade give more significance to study Atg8 lipidation, not only to complete the puzzle of autophagic pathway, but also to expand the current understanding of ubiquitin-like protein modification machineries.

Atg8 family protein lipidation cascade and diseases

As a unique protein family serving as an interactive hub to recruit other proteins to the autophagic membrane, lipidated Atg8 family members are critical in many essential biological pathways. Previous studies have implemented mouse models deficient in core autophagy genes to investigate physiological and pathogenic effects.

Previous studies in human patients showed that *ATG7* polymorphisms (SNPs) at 100A > G and 25108G > A promote the severity of asthma, likely by inducing higher level of interleukin-8 (IL-8) secretion to the serum, which potentially causes systematic inflammation (Shao et al., 2017). Moreover, changes in *ATG7* promotor region (11313449G > A, 11313811T > C, 11313913G > A and 11314041G > A) result in excessive expression of ATG7 protein, which correlates with aggravated brain damage in Parkinson disease patients (Chen et al., 2013). In addition, substitution of human ATG7 Val471 with alanine has shown association with an average 4-year earlier Huntington disease (HD) onset in European patients (Metzger et al., 2010).

Beside the E1 ATG7, all three compartments of the E3 Atg12–Atg5–Atg16 harbor significant functions in a wide collection of diseases including asthma, lupus, autoimmune disorder, bone disease, neuron degeneration, and cancer. The SNP localized in *ATG5* putative promotor region (rs12201458) has been shown negative impacts on asthma prognosis, probably due to an increase in the total ATG5 level in nasal mucosal cells, which has been observed in both acute and long-term asthma patients (Martin et al., 2012). Two of the *ATG5* SNPs (rs6937876 and rs548234) were also linked to multiple risk factors in systemic lupus erythematosus (SLE) disease onset (Qi et al., 2018; Zhou et al., 2011). Regarding to Behcet disease (BD), one of the *ATG5* genetic changes (rs573775) was proposed to have protective function by a study in a Chinese patient cohort (Zheng et al., 2015). Another SNP in *ATG5* (rs2245214) is associated with enhanced chance of developing Paget disease of bone (PDB) (Usategui-Martín et al., 2015). Even a subtle mutation E122D in ATG5 that leads to moderate structural changes causes ataxia (Kim et al., 2016). Moreover, studies in clinical patients also linked several *ATG5* SNPs with higher hepatocellular carcinoma (HCC) risk (Shen and Lin, 2019). Atg16 also plays an indispensable role in numerous disease onset or progression. For example, different SNPs occurring at the same site of human *ATG16L1* (rs2241880, T300), can confer various outputs in patient studies (Chen et al., 2015; Li et al., 2017a; Usategui-Martín et al., 2015). Change of the local deoxynucleotide to A, with an unchanged coding for T300, is related to increased risk of chronic obstructive pulmonary (COPD) (Chen et al., 2015). *ATG16L1* C allele mutation at this site was shown associated with Paget disease of bone (Li et al., 2017a). Additionally, the G SNP coding for T300A was linked to the likelihood of developing Crohn disease (Chen et al., 2015). However, in another study, the T300A variant of ATG16L1 was associated with decreased risk of brain metastasis of in non-small cell lung cancer patients (Li et al., 2017a). Another *ATG16L1* SNP rs4663402 has strong relation with greater hepatocellular carcinoma risk (Shen and Lin, 2019). Nonetheless, polymorphisms in *ATG12* promotor region (Li et al., 2017b) and protein coding region (Shen and Lin, 2019) result in enhanced occurrences of Parkinson disease and hepatocellular carcinoma in human respectively.

Summary and Aims

Autophagy is a fate-determining metabolic activity to recycle cellular material and to avoid cell death under unfavored external and internal conditions. During the

course of autophagy, ligation of Atg8 family protein to PE lipid on both side of the autophagosome membrane (Atg8 lipidation) serves as an indispensable and tightly regulated check point, which plays significant roles in phagophore nucleation, membrane extension, autophagosome engulfment, vacuole/lysosome fusing with autophagosome, and cargo recruiting. As such, in order to decipher autophagy in a comprehensive and detailed level, it is important for us to understand the molecular mechanism of Atg8 family protein ligation to the membrane.

In this study, we will introduce a variety of methods, including but not limited to biochemistry, structural biology and cell biology approaches, to investigate the molecular requirements for Atg8/LC3 lipidation in human as well as *Saccharomyces Cerevisiae* system. The results will potentially shed light on therapy development and drug design that target the Atg8 lipidation cascade serving for modulation of the autophagy pathway.

CHAPTER 2. PRODUCTION OF HUMAN ATG PROTEINS FOR LIPIDATION ASSAYS ¹

Introduction

In eukaryotic cells, autophagy is a tightly regulated process responsible for degrading and recycling cellular components, and eliminating toxic assemblies and pathogens by sequestering cargo within an autophagosome, which fuses with a lysosome (or vacuole in yeast) harboring hydrolytic enzymes (Klionsky, 2000; Wileman, 2013). Several key aspects of this regulation involve ubiquitin-like proteins (UBLs) in the Atg8 family, which form LC3 and GABARAP subfamilies in higher eukaryotes. LC3/GABARAP proteins have molecular weights in solution of approximately 14–17 kDa, but their electrophoretic migration is relatively accelerated upon ligation to lipids such as in autophagic membranes. LC3/GABARAP ligation to lipids (lipidation) plays numerous roles in regulation, including influencing the formation, maturation, and size of autophagosomes, as well as recruiting selected cargoes and regulatory proteins (Ichimura et al., 2000; Nakatogawa et al., 2007; Ohsumi and Mizushima, 2004; Tanida et al., 2008)

LC3 and GABARAP lipidation is orchestrated by a UBL ligation system, specifically conjugating the carboxyl group of the C-terminal glycine of LC3/GABARAP to the amine group of phosphatidylethanolamine (PE) (or potentially other lipids such as phosphatidyl serine) in autophagic membranes (Hanada et al., 2007; Ichimura et al., 2000; Klionsky, 2000; Sou et al., 2006; Tanida et al., 2004b, 2004c). This involves a specialized enzyme cascade, where ATG7 acts as the E1 and ATG3 acting as the E2 to produce covalent, reactive thioester-linked ATG3~LC3/GABARAP (“~” denotes thioester bond and “-” denotes a noncovalent complex) intermediates. Efficient lipidation requires an ATG12–ATG5–ATG16L1 multiprotein complex acting as the E3. Notably, formation of this E3-like assembly first requires a related conjugation cascade, wherein the UBL ATG12 is covalently conjugated to ATG5 by ATG7 acting as the E1 and ATG10 acting as the E2 (Hanada et al., 2007; Noda et al., 2013), and then the ATG12–ATG5 binds ATG16L1 to achieve full E3-like activity toward ATG3~LC3/GABARAP intermediates. Mutations or dysfunction of proteins in the LC3/GABARAP lipidation cascade results in severe diseases and poor prognosis in various eukaryotic organisms including humans (Cadwell et al., 2009; Kim et al., 2016; Lenz et al., 2011; Moloughney et al., 2011; Reed et al., 2015; Tindwa et al., 2015; Wu et al., 2014; Xue et al., 2010).

Given the importance of LC3 and GABARAP lipidation, and potential interest in targeting their conjugation for therapeutic purposes (Cheng et al., 2013; Puri and Chandra, 2014), purified proteins for assaying LC3/GABARAP lipidation and performing structural analyses would be useful (Kaiser et al., 2013; Metlagel et al., 2013, 2014; Nath et al., 2014; Otomo et al., 2013; Sakoh-Nakatogawa et al., 2013; Taherbhoy

¹ Reprinted from final submission with permission. Zheng, Y., Qiu, Y., Gunderson, J.E.C., and Schulman, B.A. (2017). Production of Human ATG Proteins for Lipidation Assays. In *Methods in Enzymology*, (Elsevier), pp. 97–113. [doi: 10.1016/bs.mie.2016.09.055](https://doi.org/10.1016/bs.mie.2016.09.055)

et al., 2011). However, our optimal expression and purification procedures differ between the human LC3/GABARAP lipidation components and their yeast counterparts (Qiu et al., 2013). Thus, in this chapter, we describe detailed methods for expression and purification of human ATG proteins involved in LC3/GABARAP lipidation, including ATG7, ATG3, ATG12–ATG5–ATG16L1, and a method for expressing LC3B as exemplary for the human Atg8 orthologues.

Expression of Human LC3B, ATG7, ATG3, ATG12–ATG5, and ATG16L1

General overview of protein expression and purification

LC3B (emblematic of LC3/GABARAP family members), ATG3, and ATG16L1 were expressed as GST-fusion proteins in *Escherichia coli*, whereas ATG7 and the ATG12–ATG5 conjugate were expressed through distinct methods in insect cells (Berger et al., 2004). An overview of the subsequent purification strategies is shown in **Figure 2-1**.

Expression constructs

A truncated version of LC3B, corresponding to residues 1-120 that form the activated form with a C-terminal glycine that is the site of chemical reactions (Tanida et al., 2004b, 2004c), is expressed from pGEX-4T-1 (**Table 2-1, Figure 2-2a**) in *E. coli*. Full-length ATG7 was expressed from a modified version of pFastBac, with TEV-cleavable N-terminal GST tag (pFastBac-GST) (**Table 2-1, Figure 2-2b**) for expression in insect cells. Full-length ATG3 was expressed from a modified version of pGEX-4T-1 harboring a cleavage site for TEV protease instead of thrombin between GST and ATG3 (pGEX-TEV) (**Table 2-1, Figure 2-2c**), for expression in *E. coli*. A truncated version of ATG16L1 corresponding to residues 1-69 was expressed from pGEX-TEV (**Table 2-1, Figure 2-2d**) in *E. coli*.

The ATG12–ATG5 subcomplex corresponds to a truncated version of ATG12 (residues 52–140) that comprises the ubiquitin-like domain (Hanada and Ohsumi, 2005) linked via an isopeptide bond between its C-terminus to Lys130 on full-length ATG5 (Hanada et al., 2007; Otomo et al., 2013). The linkage between ATG12 and ATG5 is achieved by their coexpression with ATG7 and ATG10 in insect cells using the biGBac system (Weissmann et al., 2016). First, four separate plasmids were generated. The coding sequences were inserted into pLIB vectors, with ATG7, and ATG10 untagged, ATG5 harboring a N-terminal TEV cleavable His-tag, and ATG12 (52–140) with a N-terminal TEV cleavable GST-tag (**Table 2-1, Figure 2-2e**). Next, GST-TEV-ATG12 (52–140), His-TEV-ATG5, ATG10, and ATG7 along with the promoter and terminator from the pLIB vectors were sequentially amplified using primers described in Weissmann et al., 2016, and coassembled into the pBIG1a vector (**Table 2-1, Figure 2-2f**) using the

Figure 2-1. Flow-chart indicating the expression system and purification strategies for production of human ATG proteins for lipidation assays.

Table 2-1. Plasmids used to produce human ATG proteins for lipidation assays.

Protein expressed	Plasmids generated	Functions
LC3B (1-120)	pGEX-4T-1-LC3B	Protein expression in <i>E. coli</i>
ATG7	pFastBac-GST-ATG7	To generate bacmid for protein expression in insect cells
ATG3	pGEX-TEV-ATG3	Protein expression in <i>E. coli</i>
ATG12 (52-140)~ATG5	pLIB-GST-TEV-ATG12	To generate pBIG1a plasmid
	pLIB-His-TEV-ATG5	To generate pBIG1a plasmid
	pLIB-ATG10	To generate pBIG1a plasmid
	pLIB-ATG7	To generate pBIG1a plasmid
	pBIG1a-ATG12-ATG5-ATG10-ATG7	To generate bacmid for protein expression in insect cells
ATG16L1 (1-69)	pGEX-TEV-ATG16L1	Protein expression in <i>E. coli</i>

Figure 2-2. Graphical representation of plasmids used in this study.

a. pGEX-4T-1-LC3B for LC3B (1–120) expression in *E. coli*. **b.** pFastBac-GST-ATG7 to construct bacmid for ATG7 expression in insect cells. **c.** pGEX-TEV-ATG3 for ATG3 expression in *E. coli*. **d.** pGEX-TEV-ATG16L1 for ATG16L1 (1–69) expression in *E. coli*. **e.** pLIB plasmid containing a GST-TEV-ATG12 (52–140), His-TEV-ATG5, ATG10, or ATG7 expression cassette to generate pBIG1a plasmid. **f.** pBIG1a-ATG12-ATG5-ATG10-ATG7 plasmid to construct bacmid for ATG12 (52–140)–ATG5 expression in insect cells.

method of Gibson Assembly (Gibson, 2011; Gibson et al., 2009; Weissmann et al., 2016).

All constructs were generated through transformation into DH5alpha (NEB), bacterial growth, minipreps, and verified by automated sequencing. The recommended antibiotic concentrations are: 100 µg/mL for ampicillin, 50 µg/mL for kanamycin and spectinomycin, 25 µg/mL for chloramphenicol, 10 µg/mL for tetracyclin, and 7 µg/mL for gentamycin.

Protein expression

Expression in *E. coli*

Plasmid was transformed into BL21-CodonPlus (DE3)-RIL *E. coli* competent cells (Agilent Technologies). Bacteria was plated on LB-agar with appropriate antibiotics and incubated at 37°C for 12–18 h. The starter culture was prepared from a single colony in 5 mL LB medium with antibiotics, and was cultured at 37°C, 200 rpm for 10–16 h. Then 5 mL of starter culture was inoculated into 1 L LB medium with appropriate antibiotics. The cells were grown at 37°C, 200 rpm, and monitored closely by measuring the absorbance at 600 nm. When OD₆₀₀ reaches 0.8–1.0, IPTG was added at a final concentration of 0.6 mM. Shaker temperature was reset to 18°C, allowing growing with shaking at 200 rpm for 16–20 h.

Expression in insect cells

For GST-thrombin-LC3B, GST-TEV-ATG7, and GST-TEV-ATG3, glutathione-affinity chromatography was directly applied to their corresponding cell lysates. To obtain GST-TEV-ATG12 (52–140)–His-TEV-ATG5-GST-TEV-ATG16L1 (1–69) complex, cell lysates from insect cells and bacteria were mixed at 1:1 volume ratio and incubated at 4°C for 10 min before processing.

I washed Glutathione Sepharose 4B (GS4B, GE Healthcare Life Sciences) 3 times with 10 column volumes of wash buffer in a chromatography column at 4°C. Alternatively, the beads could also be washed with 3 volumes of wash buffer by centrifugation at 500 x g for 5 min at 4°C, decanting the wash buffer, and repeating twice. Suggested bead volumes: 0.75 mL per 1 L bacterial culture, or 2 mL per 1 L Hi5 insect cell culture, which is appropriate for both GST and nickel beads and all species of ATG proteins described in this chapter. Wash buffer was the same as lysis buffer but without PMSF, aprotinin, leupeptin, or any other proteasome inhibitor.

Lysate was incubated with washed beads. The mixture was gently rocked at 4°C for 2 h for GS4B beads or 1 h for nickel beads to allow full binding.

Then lysate with beads were transferred into a Poly-Prep chromatography column (Bio-Rad) at 4°C. Lysate was allowed to flow through by gravity to generate an even

resin surface in column. Although the flow-through is unneeded in principle, it is a good practice to save the flow-through for troubleshooting in the event of problems.

If the lysate-bead solution is too thick to pass through the column, I would centrifuge the solution in 50 mL tubes at 500 x g for 5 min at 4°C. The supernatant was collected as flow through. Pelleted beads were gently resuspended in 10 column volumes of wash buffer and washed following last step.

Finally, resin was washed in column with 10 column volumes of wash buffer without disturbing the flat resin surface. This step was repeated 3 times.

Protein Purification

Preparation of cell lysate

Bacteria cells were harvested by centrifugation at 5900 x g for 12 min at 4°C. Insect cells were harvested at 500 x g for 15 min at 4°C.

Cell pellet was suspended in 15 mL lysis buffer per liter bacteria culture or 50 mL per liter insect cell culture. Lysis buffer for all proteins except the ATG12–ATG5 subcomplex was: 25 mM Tris–HCl pH 7.6, 200 mM NaCl, 2.5 mM phenylmethanesulfonyl fluoride (PMSF), 5 mM 2-Mercaptoethanol (BME). Lysis buffer for ATG12–ATG5 complex was: 1 x PBS, 400 mM NaCl, 2.5 mM PMSF, 5 mM BME. Also add 20 mg/L aprotinin, 10 mg/L leupeptin, or a protease inhibitor cocktail of choice to insect cell lysis buffer.

Bacteria cells were lysed by sonicating 7 times, or insect cells 3 times, 10 s each time with intermittent cooling on ice.

Lysate was cleared by centrifugation at 32,000 x g for 1 h at 4°C. The supernatant was collected and kept on ice.

Glutathione-affinity chromatography for LC3B, ATG7, ATG3, and ATG12–ATG5–ATG16L1

For GST-thrombin-LC3B, GST-TEV-ATG7, and GST-TEV-ATG3, glutathione-affinity chromatography was directly applied to their corresponding cell lysates. To obtain GST-TEV-ATG12 (52–140)–His-TEV-ATG5-GST-TEV-ATG16L1 (1–69) complex, cell lysates from insect cells and bacteria were mixed at 1:1 volume ratio and incubated at 4°C for 10 min before processing.

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The lysate was mixed with washed beads. The mixture was gently rocked at 4°C for 2 h for GS4B beads or 1 h for nickel beads to allow full binding.

Lysate with beads were transferred into a Poly-Prep chromatography column (Bio-Rad) at 4°C. Lysate were allowed to flow through by gravity to generate an even resin surface in column. Although the flow-through is unneeded in principle, it is a good practice to save the flow-through for troubleshooting in the event of problems.

If the lysate-bead solution is too thick to pass through the column, I would centrifugate the solution in 50 mL tubes at 500 x g for 5 min at 4°C. The supernatant was collected as flow through. Pelleted beads was gently resuspended in 10 column volumes of wash buffer and follow last step.

The resin was washed in column with 10 column volumes of wash buffer without disturbing the flat resin surface. This step was repeated 3 times.

Protease-mediated elution from beads for purification of LC3B, ATG3, and ATG7

Rough protein concentration of bead turbid solution was measured using Bio-Rad protein assay with a standard curve. Bovine serum albumin is a suitable standard. 10 µL of uncleaved sample was stored as a negative control for cleavage efficiency check before adding protease.

Thrombin protease was added to LC3B (1 mg thrombin per 100 mg of total protein), or TEV protease to ATG7 and ATG3 (2 mg TEV per 100 mg total protein). The mixture was kept gently rotated on a rocking shaker overnight to allow full cleavage. To be certain that all desired protein is cleaved from the GST-tag, I examined the bead slurry by SDS-PAGE, using a 15% acrylamide gel and Coomassie-staining, comparing cleaved samples to uncleaved samples (LC3B: **Figure 2-3**, Lanes a and b; ATG7: **Figure 2-4**, Lanes a and b; ATG3: **Figure 2-5**, Lanes a and b).

The slurry was transferred into an empty Poly-Prep chromatography column pre-rinsed with wash buffer to collect flowthrough. When liquid completely passes through, another 1 column volume of wash buffer was applied on the top of resin. This second fraction containing residual protein was also collected.

Figure 2-3. 15% SDS-PAGE showing LC3B (1–120) at different steps of purification.

Lane M shows the molecular weight markers. Lane a shows constituents of bead slurry after binding GS4B resin. Lane b shows constituents of bead slurry after thrombin treatment. Lane c shows final purified LC3B (1–120) after gel filtration chromatography.

Figure 2-4. 15% SDS-PAGE showing ATG7 at different steps of purification. Lane M shows the molecular weight markers. Lane a shows constituents of bead slurry after binding GS4B resin. Lane b shows constituents of bead slurry after TEV treatment. Lane c shows final purified ATG7 after gel filtration chromatography.

Figure 2-5. 15% SDS-PAGE showing ATG3 at different steps of purification. Lane M shows the molecular weight markers. Lane a shows constituents of bead slurry after binding GS4B resin. Lane b shows constituents of bead slurry after TEV treatment. Lane c shows purified ATG3 after anion ion exchange chromatography. Lane d shows final purified ATG3 after gel filtration chromatography.

Glutathione-mediated ATG12–ATG5-ATG16L1 elution from GS4B

0.7 resin volume of GS4B elution buffer was gently applied on the top of resin surface while keeping stopcock closed. After a 10-min incubation, I opened the stopcock and collected the eluate in a tube. Steps above were repeat 6 times with elution fractions kept in separate tubes. GS4B elution buffer: wash buffer supplemented with 20 mM glutathione (reduced, free acid) (EMD Millipore), pH adjusted by NaOH solution to the same as wash buffer.

Each eluted fraction were run on a 15% SDS-PAGE gel to determine which fractions contain the majority of target protein. Desired fractions were then pooled.

Nickel-affinity chromatography, imidazole-mediated elution from nickel beads, and TEV cleavage to obtain ATG12 (52–140)–ATG5-ATG16L1(1-69) complex

After glutathione chromatography and glutathione mediated elution, the excessive GST-TEV-ATG16L1 (1-69) was further removed by nickel affinity chromatography.

HIS-Select Nickel Affinity Gel (Sigma-Aldrich) was washed 3 times with 10 column volumes of wash buffer in a chromatography column at 4°C. Then nickel-affinity chromatography was performed to enrich the ATG5-bound complex. The glutathione elution of GST-TEVATG12 (52–140)-His-TEV-ATG5-GST-TEV-ATG16L1 (1-69) was applied to the HIS-Select Nickel Affinity Gel beads and was incubated following the same procedure described above for glutathione-affinity chromatography. An imidazole elution was performed following steps described in glutathione elution using nickel bead elution buffer (wash buffer supplemented with 100 mM imidazole pH 7.5). 10 µL uncleaved sample was stored as a negative control to check cleavage efficiency.

TEV protease (2 mg TEV per 100 mg total protein) was added to the imidazole eluate. Then the eluate was dialyzed in wash buffer while performing cleavage overnight at 4°C. Cleavage completeness was examined by SDS-PAGE, using a 15% acrylamide gel and Coomassie-staining (**Figure 2-6**, Lanes a and b). The cleaved GST and HisMBP tags were removed from ATG12 (52–140)–ATG5-ATG16L1 (1-69) complex by further cation ion exchange purification described below.

Ion exchange chromatography on ATG3 and ATG12(52–140)~ATG5-ATG16L1 (1-69)

Anion ion exchange was performed to tag-free ATG3 and cation ion exchange to tag-free ATG12 (52–140)–ATG5-ATG16L1 (1-69) accordingly to their isoelectric points, before ultimately purifying by gel filtration chromatography. This step is optional for LC3B and ATG7.

Figure 2-6. 15% SDS-PAGE showing ATG12 (52-140)-ATG5-ATG16L1 (1-69) complex at different stages of purification.

Lane M shows the molecular weight markers. Lane a shows elution from nickel-affinity chromatography, capturing His- and His-MBP tags on ATG5 and ATG16L1 (1-69), respectively. This is the second step in the purification, following an initial purification by glutathione affinity chromatography to capture GST-tag on ATG12. Lane b shows product of TEV cleavage reaction preformed during dialysis. Lane c shows final purified ATG12 (52-140)-ATG5-ATG16L1 (1-69) complex after subsequent cation ion exchange and gel filtration chromatography.

A 5 mL HiTrap SP (cation exchange) or a Q (anion exchange) HP column (GE Healthcare Life Sciences) was equilibrated on an AKTA FPLC (GE Healthcare Life Sciences) in 95% buffer A with 5% buffer B. Cation exchange buffer A: 25 mM HEPES pH 7.0, 5 mM DTT. Anion exchange buffer A: 25 mM Tris-HCl pH 8.0, 5 mM DTT. Buffer B corresponded to buffer A with additional 1 M NaCl. The protein samples were diluted with buffer A to a final NaCl concentration of 50 mM. Samples were clarified by centrifugation at 4000 x g for 5 min at 4°C before loading onto a FPLC system. If loading is through a sample pump, I would equilibrate the sample pump in buffer A. The ATG proteins described in this chapter can be concentrated to 10 mg/mL if desired to reduce volume prior to loading onto FPLC.

After equilibration, the sample was loaded onto column with a suggested loading rate of 2 mL/min. After loading, the column was washed by 5 column volumes of 95% buffer A with 5% buffer B. After all unbound sample was washed out, I initiated gradient, raising buffer B proportion from 5% to 50% over 25 column volumes at 2 mL/min flow rate, and collecting fractions with a volume of 2 mL.

Peak fractions were examined by SDS-PAGE with Coomassie-stained 15% acrylamide gels to determine which fractions contain the majority of the ATG proteins (the combined fractions of ATG3 is shown in **Figure 2-5**, Lane c; ATG12-ATG5-ATG16L1 is not shown).

Gel filtration chromatography for LC3B, ATG7, ATG3, and ATG12-ATG5-ATG16L1

Superdex 200 10/300 GL columns (GE Healthcare Life Sciences) were used for all the ATG proteins described here, except for LC3B using a Superdex 75 10/300 GL column (GE Healthcare Life Sciences). Protein samples of LC3B and ATG7 used in this step were directly collected from protease cleavage, while ATG3 and ATG12-ATG5-ATG16L1 were pre-purified by ion exchange.

A Superdex 75/200 column was equilibrated on an AKTA FPLC system with storage buffer (25 mM Tris-HCl pH 7.6, 200 mM NaCl, 5 mM DTT). NaCl concentration was increased to 500 mM in the buffer used for LC3B, or to 300 mM in the buffer used for ATG12 (52-140)-ATG5-ATG16L1 (1-69).

Then the protein samples were concentrated by Amicon Ultra Centrifugal Filter Units (EMD Millipore) with appropriate molecular weight cutoff. Resulting concentrated protein samples were transferred to one or multiple 1.7 mL microcentrifuge tubes. Centrifugation was performed at 16,000 x g for 10 min at 4°C to clarify samples before loading onto a FPLC system.

1-2 mL clarified protein sample was loaded onto a Superdex 200 column, followed by gel filtration run in storage buffer. The fractions were collected in 0.5 mL volume size.

The peak fractions were examined by SDS-PAGE with Coomassie-stained 15% acrylamide gels to determine which fractions contain the majority of the ATG proteins. Desired fractions were pooled (the pooled fractions of LC3B, ATG7, ATG3, and ATG12–ATG5–ATG16L1 are shown in **Figure 2-3**, Lane c; **Figure 2-4**, Lane c; **Figure 2-5**, Lane d; **Figure 2-6**, Lane c, respectively).

Storing Purified Proteins

Purified proteins was concentrated to approximately 10 mg/mL and stored in 20 μ L aliquots at - 80°C after flash-freezing in liquid nitrogen. They can be thawed on ice for use and refreezed for storage up to twice to ensure protein activity.

Concluding Remarks

Proteins prepared as described earlier are eligible for multiple biochemical assays, including LC3B lipidation. In our experience, aliquots of purified ATG proteins stored at - 80°C are stable for up to a year, giving consistent results in assays for LC3B lipidation, formation of thioester bonded ATG7~LC3B and ATG3~LC3B intermediates, binding using BioLayer Interferometry (Octet Red, ForteBio) or isothermal titration calorimetry (ITC), and for structural studies using NMR and crystallography.

CHAPTER 3. A SWITCH ELEMENT IN THE AUTOPHAGY E2 ATG3 MEDIATES ALLOSTERIC REGULATION ACROSS THE LIPIDATION CASCADE ²

Introduction

The central enzyme in the Atg8 lipidation cascade is the E2, Atg3 (Klionsky, 2000; Wileman, 2013). Atg3 receives Atg8 from the E1 Atg7, to form a covalent, reactive Atg3~Atg8 intermediate linked by a thioester bond (referred to by ~) between the C-terminus of Atg8 and the catalytic Cys of Atg3. Atg8 is then transferred from the Atg3 catalytic Cys to PE in a reaction catalyzed by a composite E3 enzyme (Fujita et al., 2008b)4/15/2020 10:13:00 AM containing another autophagy UBL, Atg12, conjugated to Atg5 (Hanada et al., 2007). *In vitro*, the yeast Atg12–Atg5 conjugate is sufficient to serve as an E3 enzyme activating lipidation of Atg8 (Hanada et al., 2007). Atg16 binding to lipids substantially enhances E3 activity (Lystad et al., 2019) and is required *in vivo* (Mizushima et al., 1999) to localize Atg12–Atg5 to the preautophagosomal structure that precedes autophagosome formation (Kim et al., 2001; Suzuki, 2001), through many mechanisms (Harada et al., 2019; Kaufmann et al., 2014; Romanov et al., 2012).

The prior structure of Atg3 from *S. cerevisiae* revealed a catalytic core domain (Atg3^{cat}) largely resembling E2s for canonical UBLs such as Ub, NEDD8 and SUMO, including a long central helix and two catalytic segments (Yamada et al., 2007) (**Figure 3-1a**). One segment harbors the catalytic Cys234, and the other displays Thr213, which also is essential for the lipidation reaction and corresponds to the canonical E2 catalytic Asn that plays a crucial structural role (Berndsen et al., 2013; Ichimura et al., 2000; Sakoh-Nakatogawa et al., 2013). In addition, Atg3 displays unique, functionally-important structures (Nath et al., 2014; Yamada et al., 2007; Yamaguchi et al., 2010): an N-terminal amphipathic membrane-binding helix (N, residues 1-18); an intrinsically-disordered flexible region (FR, residues 85-159); a handle region consisting of a helix and a disordered region (HR, residues 237-282) that contains an Atg8-interacting motif that is dispensable for the lipidation reaction but plays important roles *in vivo*; and a flexible extreme C-terminal extension (C, residues 299-310). While prior structural studies showed how Atg7 engages Atg3^{cat} and the Atg3^{FR} for Atg8 transfer to Atg3 (Hong et al., 2011; Noda et al., 2011; Taherbhoy et al., 2011; Yamaguchi et al., 2012), less is understood about subsequent roles of the autophagy E3. A crystal structure showed how the human E3 enzyme recruits a peptide-like region from Atg3's FR, but this FR motif is not conserved across Atg3 sequences from many organisms, including yeasts (Metlagel et al., 2013). Furthermore, the structure lacked Atg3's catalytic domain, for which prior studies of cysteine accessibility in the yeast enzyme suggested that the autophagy E3 triggers a structural rearrangement that affects the orientation of the

² Reprinted from final submission with open access permission. Zheng, Y., Qiu, Y., Grace, C.R.R., Liu, X., Klionsky, D.J., and Schulman, B.A. (2019). A switch element in the autophagy E2 Atg3 mediates allosteric regulation across the lipidation cascade. *Nature Communications* 10, 1–14. <https://doi-org.ezp-prod1.hul.harvard.edu/10.1038/s41467-019-11435-y>

Figure 3-1. The Atg12-Atg5 module within the autophagy E3 activates intrinsic reactivity of the Atg3~Atg8 intermediate.

a, Structures of yeast Atg3 (left, PDB 2DYT), a ubiquitin E2 (middle, UBE2D1, PDB 4AP4), and their superimposition (right). Atg3 unique elements are indicated by colors shown in schematics of Atg3 constructs used in this study (below). **b**, Intrinsic reactivity of Atg3~Atg8 intermediate monitored by pulse-chase discharge to NH_2OH in presence of indicated E3 variant. **c**, Close-up of structural superimposition of yeast Atg12-Atg5-Atg16 (PDB 3W1S) and corresponding human complex with ATG3^{FR} (PDB 4NAW). Residues from yeast Atg12 corresponding to the human ATG3^{FR}-binding site are shown as red sticks. **d**, Effects of mutations in minimal yeast E3 (Atg12-Atg5) on stimulating Atg3~Atg8 discharge, monitored by quantification of Atg3~Atg8 remaining after 2.5 minutes in pulse-chase assays using the scheme as in b, as a function of NH_2OH concentration. (Error bar: STDEV, N = 3) Representative gel is shown in Supplemental Figure 3-2b.

catalytic Cys234 relative to Thr213 (Sakoh-Nakatogawa et al., 2013). The molecular underpinnings for E3-dependent conformational activation remains elusive.

Here we use a hybrid structural approach integrating information from biochemistry, NMR, crystallography, and yeast genetics to identify a molecular basis for Atg3 allosteric activation. Overall, the data suggest that a distinct peptide-like element acts like a brace mediating intramolecular interactions with the Atg3 catalytic domain to restrict the conformation of the active site and prevent reactivity toward errant nucleophiles, while the E3 elicits an unprecedented E2 activation mechanism by remotely binding to this brace to couple other E3 interactions with allosteric activation of active site mediating Atg8 lipidation.

Methods

Protein sequence alignment

ScAtg3 and HsATG3 protein sequence alignment are done by CLUSTALW online.

Protein expression and purification

Detailed methods for cloning, expressing and purifying yeast proteins Atg8, Atg3 (and their mutants or truncated versions) and Atg16 (residues 1-46) were described previously (Kaiser et al., 2013; Taherbhoy et al., 2011; Zheng et al., 2017). For Atg7 proteins, full-length Atg7 or a fragment containing residues 1-289 (NTD) was inserted into the pGEX-4T-1 vector following the Gibson Assembly protocol (Gibson et al., 2009), and transformed into BL21 competent *E. coli*; protein expression and purification protocols for Atg7 WT and mutants resemble those for Atg3 and Atg8. To obtain purified E3 complex, N-term 6X His-tagged Atg12 100-186 and untagged Atg5 1-284 were inserted into the two multiple cloning sites (MSCs) of pRSF-Duet-1 respectively, whereas Atg7 1-630 and Atg10 were inserted into the MSCs of pET-Duet-1. The two plasmids are co-transformed into BL21 competent *E. coli* to produce His-Atg12-Atg5. Bacteria culture were grown at 37 °C to reach O.D. 1.5, followed by 0.6 mM IPTG induction at 23 °C overnight. Cells are harvested in 1X PBS, 400 mM NaCl and lysed by sonication. Ni-NTA resin (Qiagen) was incubated with lysate for 1 hour then washed 3 times with the same buffer. His-Atg12-Atg5 were eluted by buffer with 200 mM imidazole and immediately purified by cation exchange chromatography on an S column (GE Healthcare). Purified his-Atg12-Atg5 were combined with Atg16 1-46 in a 1:2 molar ratio and further purified by size-exclusion chromatography on a Superdex-200 column (GE Healthcare) to harvest peak fractions containing the final product of His-Atg12-Atg5-Atg16.

Crystallization and data collection

1 μL purified Atg3 ^{ΔNFR} protein sample was manually loaded onto a glass hanging-drop cover and overlaid with 1 μL of precipitant solutions. Crystal was harvested from 100 mM HEPES pH 7.2, 150 mM L-malic acid, 9% PEG3350. Crystals were allowed to grow at 4 °C for 5-7 days to reach their maximum sizes. All crystals were harvested by loops with 25% glycerol in addition to growth solution, and stored in liquid nitrogen for 2 days until data collection.

Data collection, processing, and structure refinement

Data for Atg3 ^{ΔNFR} was collected at Advanced Photon Source (APS) Ser-CAT beamline 22-BM. The Dataset was created from single crystals, and was processed and scaled in HKL2000. Phase was solved by molecular replacement. Search model for Atg3 ^{ΔNFR} was modified from Atg3^{FL} (PDB 2DYT) with Atg3^{E123IR} manually removed. Model was built in Coot (Emsley et al., 2010), and refinements were primarily performed in Refmac (Murshudov et al., 1997) and finalized in Phenix (Adams et al., 2010).

Simulated annealing omit map generation

In order to avoid the model bias, we generated a simulated annealing omit map for the catalytic cysteine region. First, residue 231-237 in the final-refined model was deleted; then, the model with deletion was subjected to 3 rounds of refinement in Phenix⁶² with simulated annealing running from 5000K to 300K in 50 steps. Finally, the Fo-Fc map was shown at 3 σ level along with the deleted residues in the model.

Atg3 C234 and Atg8 G116C disulfide crosslinking

All the cysteines except C234 within Atg3 were mutated to alanines. 800 μM Atg8 K26P C22V G116C was reduced by 20 mM DTT, desalted into buffer 1 (20 mM HEPES pH 7.5, 50 mM NaCl), immediately mixed with 1:1 v/v buffer 2 (20 mM HEPES pH 7.5, 50 mM NaCl, 2.5 mM 2,2'-dipyridyldisulfide (DPS)). DPS stock was pre-dissolved in a minimal amount of DMSO, incubated at room temperature (R.T.) for 20 min, and desalted again into buffer 1. Meanwhile, 500 μM Atg3 was reduced by 20 mM DTT and desalted into buffer 1, immediately mixed with DPS-Atg8 at a 1:2 molar ratio, and incubated at R.T for 1 hour. Crosslinking product was purified by anion exchange chromatography on Q column (GE Healthcare) and size-exclusion chromatography on a Superdex-200 column (GE Healthcare).

NMR spectroscopy

All the experiments were conducted at 25 °C with protein samples dissolved in 20 mM MES pH 6.5, 100 mM NaCl, and 10% v/v D₂O on either a Bruker Avance 600-MHz

or 700-MHz or 800-MHz spectrometer equipped with a 5-mm triple resonance cryoprobe and a single-axis pulse field gradient. For resonance assignments of backbone atoms, 3D triple-resonance experiments were measured either with 0.4 mM ¹³C, ¹⁵N, ²D-labeled or ¹³C, ¹⁵N-labeled Atg3^{cat}, 0.75 mM ¹³C, ¹⁵N-labeled Atg3^{FR} 86-159, and 0.4 mM ¹³C, ¹⁵N-labeled Atg8 K26P, C33V, G116C. The backbone assignment of Atg3^{cat} was carried out using 3D HNCACB, HNcoCACB, HNCO, HNcaCO along with ¹⁵N-resolved [¹H, ¹H] NOESY spectra. The backbone of Atg3^{FR} 86-159 fragment peptide was assigned using 3D HNCACB, CACBcoNH, HNCO, HNcaCO along with HNcoNH spectra. The backbone assignment of Atg8 K26P, C33V, G116C was carried out using 3D HNCACB, HNcoCACB, HNCO, HNCA and HNcoCA along with ¹⁵N-resolved [¹H, ¹H] NOESY spectra. Spectra were processed with Topspin 3.5 or nmrPipe and analyzed using CARRA. All chemical shift perturbation analyses were done using [¹⁵N, ¹H] TROSY spectra, and the perturbations were calculated using the formula CSP (ppm) =

$((\delta H)^2 + 0.2 * (\delta N)^2)^{0.5}$. Titration experiments were done using 0.1 mM ¹⁵N-labeled Atg3^{FR} 86-159 (or its I132D, L135D, I136D mutant) titrated with 0-150 μM Atg12–Atg5, 0-500 or 0-625 μM Atg3^{ΔFR}, or 0 and 0.2 mM unlabeled Atg3^{ΔFR} C234~Atg8 K26P, C33V, G116C disulfide complex; 0.1 mM ¹⁵N-labeled Atg3^{cat} was titrated with 0 or 4.4 mM unlabeled Atg3^{FR} 123-147. Also spectrum from 0.1 mM ¹⁵N-labeled Atg8 was compared with 0.1 mM disulfide complex of unlabeled Atg3^{cat} cross-linked via C234 to ¹⁵N-labeled Atg8 K26P, C33V, G116C. The CSP value is used in the titration plots to get the K_d values using the equation: $y = (y_0 + x) / (x + K_d)$, where x is the concentration and K_d is the dissociation constant and y₀ is a constant.

Since most of the resonances in Atg3^{FR} mutant [I132D, L135D, I136R] have the same chemical shifts as that of Atg3^{FR}, the assignment of Atg3^{FR} was used to assign the resonances of Atg3^{FR} mutant. The assignment of the mutated residues and the neighboring ones that shifted the most ambiguously shifted to positions where new peaks appeared.

Liposome preparation

2.5 mg of *E. coli* polar lipid extract (Avanti) was dissolved in chloroform, solvent evaporated using a nitrogen stream, and re-dissolved in 250 μL 20 mM HEPES pH 7.0, 150 mM NaCl buffer. Product was passed through 100 nm nuclepore membrane (Whatman) installed in a mini extruder (Avanti) 15 times to obtain 10 mg/mL homogenized liposomes in solution.

***In vitro* pulse-chase assay for Atg3~Atg8 discharge to NH₂OH**

Shortly before the starting the experiment, Atg3 and mutants were reduced with 20 mM DTT and desalted by Zeba columns (Thermo Fisher Scientific) into reaction buffer. In the pulse step, 5 μ M Atg7, 20 μ M Atg3, 40 μ M Atg8, 1 μ M ATP and 1 μ M MgCl₂ were mixed in 25 μ L 20 mM HEPES pH 7.0, 150 mM NaCl buffer. After 1 hour, pulse reactions were quenched by adding Apyrase. In chase step, 5 μ L of each pulse product was incubated with 5 μ L chase solution containing 5 μ M Atg12–Atg5–Atg16 and a course of NH₂OH at R.T. for 2.5 minutes, or with liposome at a final concentration of 2 mg/mL added in a timely manner. Samples were analyzed by 4%-12% Bis-Tris Protein Gels (Thermo Fisher Scientific) and stained with Coomassie Brilliant Blue. All raw data are available in the source data file.

***In vitro* assay for Atg8 lipidation**

Atg3 and mutants were reduced and desalted as described in the pulse-chase assay. Reactions were performed at 30 °C in 25 μ L volumes of 20 mM HEPES pH 7.0, 150 mM buffer, with 1 μ M Atg7, 2 μ M Atg3, 5 μ M Atg8, 5 μ M Atg12–Atg5–Atg16, 1 mM ATP, 1 mM MgCl₂ and 1 mg/mL liposome, and were monitored in timely manner. 6 μ L of each reaction was mixed with 6 μ L 2X protein loading buffer. Samples were analyzed using a by 15 % urea gel at 4 °C and stained with Coomassie Brilliant Blue. All raw data are available in the source data file.

Yeast strains, vectors, media and growth conditions

The XLY161 and the YCY131 (Cao et al., 2009) cells were transformed with pRS416 vectors harboring HA-tagged Atg3 (WT or mutants); the YCY131 cells were co-transformed with pRS414-Atg7-Atg10-Atg8 Δ R. For nutrient-rich conditions, yeast cells were grown in synthetic minimal (SMD; 0.67% yeast nitrogen base, 2% glucose, and auxotrophic amino acids and vitamins as needed) medium. For nitrogen starvation, cells were first cultured in appropriate SMD medium to mid-log phase; they were then shifted to the SD-N medium (0.17% yeast nitrogen base without ammonium sulfate or amino acids, 2% glucose). The XLY161 cells were cultured in SMD-Ura to mid-log phase before they were shifted to SD-N medium for 2 h; The YCY131 cells were cultured in SMD-Ura-Trp to mid-log phase before they were shifted to SD-N medium for 4 h. All raw data are available in the source data file.

Western blot

1 OD of each of the yeast cultures was harvested and lysed in 50 μ L MURB (50 mM NaH₂PO₄, pH 7.0, 2.5 mM MES, pH 7.0, 1% SDS, 3 M urea, 0.5% β -mercaptoethanol, 1 mM NaN₃, 0.2 μ g/ μ L bromophenol blue) by vortexing them with glass beads at 4°C for 5 min. The samples were then heated at 70°C for 10 min before

they were loaded onto and run in SDS-PAGE gels, followed by transferring onto PVDF membranes. The membranes were blocked with 5% skim milk, probed with primary antibodies (1:3000, Atg8; 1:10000, Dpml; 1:3000, HA; and 1:10000, Pgk1), and then probed with horseradish peroxidase (HRP)-coupled secondary antibodies (1:10000, anti-rabbit and anti-mouse antibodies). Atg8 antiserum was generated by injecting purified yeast Atg8 peptides into rabbits; Dpml antibody is purchased from Molecular Probes (catalog# A-6429); antibody to the HA epitope is from Sigma (catalog# H3663); Pgk1 antibody is a generous gift from Dr. Jeremy Thorner, University of California, Berkeley. Goat anti-rabbit HRP secondary antibody is from Fisher (catalog# ICN55676), and rabbit anti-mouse HRP secondary antibody is from Jackson ImmunoResearch (catalog# 315-035-003). Chemiluminescence images were obtained by developing films in a dark room. The standard-sized protein bands from the protein ladder lanes on the blots were correspondingly hand-drawn using a pen onto the developed films. The hand-drawn protein ladders were not shown in the figures, but they can be found in the corresponding source files.

Results

Autophagy E3 activates intrinsic reactivity of Atg3~Atg8

E2~UB and E2~UBL intermediates are activated in distinct ways by different classes of E3 enzymes (Buetow and Huang, 2016; Cappadocia and Lima, 2018; Zheng and Shabek, 2017). Many E3s, for example RING and related E3s for UB, NEDD8 and SUMO, and SIM-containing SUMO E3s, allosterically promote noncovalent interactions between the E2 and UB (or UBL) moieties within the covalent intermediate. This is often monitored by an E3 increasing the susceptibility of an E2~UB or E2~UBL intermediate to nucleophilic attack by a nonspecific amine nucleophile (Saha and Deshaies, 2008). In contrast, HECT and RBR E3s use a different mechanism that depends on positioning the E2-bound UB for transfer to the E3 catalytic Cys. Although the autophagy E3 lacks homology to UB, NEDD8, and SUMO ligases, we asked whether there are common mechanistic features by testing biochemical activities of the relatively simplistic Atg8 pathway from *S. cerevisiae*. Potential E3-mediated stimulation of intrinsic activity of the Atg3~Atg8 intermediate was examined by adapting the pulse-chase assay format used to study canonical UB enzymes (**Figure 3-1b**). Briefly, a pulse-reaction catalyzed by Atg7 generated the thioester-linked Atg3~Atg8 intermediate. After quenching this reaction with apyrase, increasing concentrations of hydroxylamine were added (**Figure 3-1b**). Discharge of the Atg3~Atg8 intermediate to this nonspecific amine nucleophile was monitored by appearance of free Atg3 in SDS-PAGE. Whereas the Atg3~Atg8 intermediate was relatively stable on its own, addition of Atg12~Atg5 stimulated discharge. This activation was maintained in the presence of Atg16, while Atg5~Atg16 alone was insufficient (**Figure 3-1b**). Atg12~Atg5 is also known to be required for E3 activation of the lipidation reaction (Hanada et al., 2007; Lystad et al., 2019; Sakoh-Nakatogawa et al., 2013). Thus, within the composite autophagy E3 is a module that activates the intrinsic reactivity of the Atg3~Atg8 thioester-linkage.

As a first step toward understanding the mechanism, we performed mutagenesis based on the prior structure showing that in the context of the human autophagy E3, Atg12 recruits a short peptide-like region of Atg3's FR (Metlagel et al., 2013). Although the FR sequences from human and yeast Atg3 are not conserved (**Figure 3-2a**), the structures of human and yeast Atg12–Atg5 superimpose well, including the Atg3^{FR}-binding site (**Figure 3-1c**) (Noda et al., 2013; Otomo et al., 2013). Mutations in the corresponding surface of yeast Atg12–Atg5 not only impair Atg8 lipidation but also intrinsic activation of the Atg3~Atg8 intermediate (**Figure 3-1d** and **Figure 3-2b,c**). Thus, it seems probable that the FR from yeast Atg3 likewise contains an E3-binding element.

Identification of an E3-binding element in yeast Atg3

A multi-tiered approach was used to identify the Atg3 element determining E3 activation and binding. First, given that the corresponding E3-binding element from human Atg3 is located in the FR, a panel of Atg3 variants harboring individual or multiple alanine mutations in the FR were monitored for E3-dependent Atg3~Atg8 discharge (**Figure 3-3a** and **Figure 3-4**). Second, mutants hindering discharge were tested for relevance to autophagy, by testing for effects on E3-dependent Atg8 lipidation *in vitro* and *in vivo* (**Figure 3-3b,c**). Finally, NMR was used to define the Atg3^{FR} region mediating E3-binding. After resonances were assigned for a construct spanning the entire intrinsically-disordered Atg3^{FR} (residues 86-159), an unlabeled version of the Atg12–Atg5 conjugate was added, and [¹⁵N, ¹H] TROSY spectra were examined for chemical shift perturbation and/or line-broadening as an indicator of binding (**Figure 3-5a,c**). Although other residues in Atg3's FR contribute to activity (**Figure 3-3a**), the major region implicated by all assays spans from Ile129-Lys142 (**Figure 3-3a-c** and **Figure 3-5a,c**). For reasons described below, we termed this region Atg3^{E123IR}.

Atg3's E3-binding element also binds E1 and E2

Prior studies showed that the region corresponding to Atg3^{E123IR} mediates Atg3 recruitment to the autophagy E1, Atg7 (Noda and Klionsky, 2008; Taherbhoy et al., 2011). In this upstream step of the conjugation cascade, Atg3^{E123IR} binding to Atg7's N-terminal domain (Atg7^{NTD}) anchors the complex, thereby increasing the local concentration for distal, transient interactions between Atg7's and Atg3's catalytic domains for Atg8 transfer between them (Kaiser et al., 2013) (**Figure 3-6a**). To further confirm that as in the human autophagy pathway (Ohashi and Otomo, 2015; Qiu et al., 2013), E1 and E3 binding to Atg3 is mutually exclusive for yeast enzymes, we examined the ability of the isolated NTD from Atg7 to compete with Atg12–Atg5-dependent activation of Atg3~Atg8. The wild-type Atg7^{NTD} inhibits the E3-dependent reaction, whereas a version harboring a mutation (P283D) previously shown to impair Atg3 binding (**Figure 3-6a**) (Taherbhoy et al., 2011) does not show this inhibitory effect, consistent with mutually-exclusive binding (**Figure 3-6b**).

Figure 3-2. Lack of sequence conservation for Atg3 FR yet functional conservation of FR-binding site on Atg12 within Atg12-Atg5 E3 module.
a, Sequence alignment of Atg3 from *S. cerevisiae* (yeast) and *H. sapiens* (human). Elements in yeast Atg3 are labeled. **b**, Representative gel image of 3 times repeated assays from **Figure 3-1d**. **c**, Effects of indicated Atg12 Ala mutants in predicted FR-binding site from Atg12-Atg5 on Atg8 lipidation in vitro, in reactions with Atg7, Atg12-Atg5-Atg16, and liposomes generated from *E. coli* polar lipids as a source PE, and detected by migration of Atg8 in Coomassie-stained SDS-PAGE gel.

Figure 3-3. E123IR plays essential role in E3-dependent Atg8 ligation activity.
a, Effects of indicated Ala mutants in Atg3's FR on E3 (Atg12–Atg5–Atg16) activation of Atg3~Atg8 intermediate, quantified as percent Atg3~Atg8 remaining in pulse-chase discharge to NH₂OH over 2.5 minutes. Locations of most defective and moderately defective mutants are indicated on schematics, and those corresponding to Atg3^{E123IR} are shown in red. **b**, Effects of indicated Ala mutants in Atg3's FR on Atg8 lipidation *in vitro*, in reactions with Atg7, Atg12–Atg5–Atg16, and liposomes generated from *E. coli* polar lipids as source of PE, and detected by migration of Atg8 in Coomassie-stained SDS-PAGE gel. **c**, Effects of indicated Ala mutants in Atg3's FR on Atg8 lipidation *in vivo*, as detected by western blot for Atg8 after 2 hours starvation of XLY161 *atg3Δ pep4Δ* strain of *S. cerevisiae* expressing either WT or mutant HA-tagged Atg3. Pgk1 is loading control for Atg8, Dpm1 is loading control for Atg3.

Figure 3-4. Alanine scan within FR of Atg3 for E3-dependent activation of Atg3~Atg8 intermediate.
Representative gel image of assay from **Figure 3-3a**.

Figure 3-5. E123IR interacts with autophagy E3.
a, [^{15}N , ^1H] TROSY spectra of ^{15}N -labeled Atg3^{FR} titrated with unlabeled Atg12-Atg5-Atg16. **b**, Estimated binding affinities between E123IR residues and Atg12-Atg5, based on Chemical Shift Perturbations (CSPs) observed upon titrating increasing concentrations of Atg12-Atg5. **c**, Chemical shift perturbations plotted as a function of Atg3^{FR} residue numbers, with resonances showing intermediate exchange line broadening indicated by stripes.

Figure 3-6. E123IR binds the E1 Atg7 and E2 core domain Atg3^{cat}.

a, Close-up showing interactions between Atg3^{E123IR} and N-terminal domain (NTD) of Atg7 (PDB 3T7G). **b**, Effects of Atg3^{E123IR}-binding WT Atg7 NTD and non-binding mutant (P283D) on E3-stimulated intrinsic reactivity of Atg3~Atg8 intermediate, as monitored by pulse-chase discharge to NH₂OH. **c**, [¹⁵N, ¹H] TROSY spectra of ¹⁵N-labeled Atg3^{cat} alone (red) or in 1:44 mixture with unlabeled Atg3^{FR} (cyan), with Chemical Shift Perturbations (CSPs) plotted per residue below. **d**, [¹⁵N, ¹H] TROSY spectra of ¹⁵N-labeled Atg3^{FR} titrated with unlabeled Atg3^{AFR}, with representative (1:5 molar ratio Atg3^{FR} vs Atg3^{AFR}) CSPs plotted per residue below. **e**, Estimated binding affinities between E123IR residues and Atg3^{AFR}, based on CSPs observed upon titrating increasing concentrations of Atg3^{AFR}. **f**, Residues corresponding to greatest CSPs are shown as spheres on the structure.

Intriguingly, this same E1 and E3-binding Atg3^{E123IR} element was also found to contact the Atg3^{cat} domain in the prior crystal structure of full-length Atg3 alone, although the interactions were previously attributable to crystal packing and not tested further (**Figure 3-1a**) (Yamada et al., 2007). To address the potential for bona fide interaction in solution, we wished to perform NMR. This first required generation of suitable fragments from Atg3, because in prior studies of the full-length protein, it was not possible to detect resonances from the catalytic domain due to dramatically different dynamic properties from the FR region (Yamada et al., 2007). Thus, the isolated Atg3^{cat} domain was prepared (construct listed in **Figure 3-1a**), and resonances observed in [¹⁵N-¹H] TROSY spectra were assigned (**Figure 3-6c**). Intriguingly, resonances corresponding to the active site loop (residues 232-236) were not assignable in the spectrum, potentially due to motions on the millisecond or intermediate exchange time scales.

[¹⁵N-¹H] TROSY spectra obtained upon adding a synthetic peptide corresponding to the Atg3^{FR} element to ¹⁵N-labeled Atg3^{cat} (**Figure 3-6c**), or upon adding an unlabeled version of Atg3^{ΔFR} to ¹⁵N-labeled Atg3^{FR} (**Figure 3-6d**), showed substantial chemical shift perturbations. In both titrations, the perturbed resonances correspond to the Atg3^{FR} and Atg3^{cat} domain residues observed to interact in the crystal structure (**Figure 3-6f**). Consistent with the interactions, introduction of mutations to hydrophobic residues I132, L135, and I136 significantly diminished the chemical shift perturbations within E123IR (**Figure 3-7a**), and further lower the E123IR-Atg3^{ΔFR} binding affinity (**Figure 3-7b**). Thus, the NMR data confirmed the Atg3^{E123IR} element as a bona fide Atg3^{cat}-binding element in solution. The term Atg3^{E123IR} reflects this being an E1, E2, and E3 interacting region.

Compared to the interaction with Atg12–Atg5 (**Figure 3-5b**), we observed lower binding affinity between E123IR and Atg3^{ΔFR} (**Figure 3-6e**). To gain insights into the structure of E123IR in its free form, we examined the $\Delta\delta C^\alpha - \Delta\delta C^\beta$ secondary chemical shifts, which show this element is not a helix in isolation (**Figure 3-7c**) (Marsh et al., 2006). It seems likely that a combination of different affinities for different partners, and intrinsic conformational plasticity could enable switching between E1, E2, and E3-bound states.

Active site loop conformation in absence of Atg3^{E123IR}

To illuminate roles of Atg3^{E123IR}-binding to the Atg3^{cat}, we determined a crystal structure lacking these interactions for comparison to the prior structure of full-length Atg3. The structure of a version of Atg3 lacking the N-terminal 18 residues and the FR region (Atg3^{ΔNFR}) (**Figure 3-8a**, **Figure 3-9a**, and **Table 3-1**), determined at 2.5 Å resolution, superimposes well overall with that of full-length Atg3 (0.48 RMSD over C- α s) (**Figure 3-8b**). However, there are substantial differences in the catalytic Cys loop and adjacent regions. As described previously, in the structure of full-length Atg3, the catalytic Cys234 is sequestered in a pocket formed by side chains from the HR and two loops (Yamada et al., 2007). This orientation is catalytically incompetent, with the Cys distal from the structurally-important Thr213 and from two other side-chains

Figure 3-7. Mutations in E123IR hydrophobic residues significantly impair E123IR-Atg3 interaction.

a, [^{15}N , ^1H] TROSY spectra of ^{15}N -labeled Atg3^{FR} titrated with increasing concentrations of unlabeled Atg3^{ΔFR}, with chemical shift perturbations (CSPs) plotted per residue shown below for one representative point in the titration (1:5 molar ratio Atg3^{FR} mutant versus Atg3^{ΔFR}). **b**, Calculated binding affinities between Atg3^{ΔFR} and residues in the I132D,L135D,I136D mutant version of Atg3^{FR}, based on CSPs along the titration. **c**, The $\Delta\delta\text{C}\alpha - \Delta\delta\text{C}\beta$ secondary chemical shifts of the Atg3^{FR} peptide in its free form.

Figure 3-8. Conformational changes upon E123IR removal from Atg3.
a, Crystal structure of Atg3^{ΔNFR} (PDB 6OJJ, from this study). **b**, Superposition of Atg3^{ΔNFR} (PDB 6OJJ, light blue with catalytic Cys shown in green) with prior structure of Atg3^{FL} (cyan, PDB 2DYT) with E123IR and catalytic Cys shown in red, and differences highlighted in cartoons. **c**, Fo-Fc map shown at 3σ after omitting the catalytic cysteine region (residues 231-237) of Atg3^{ΔNFR} and performing simulated annealing. **d**, Close-ups of catalytic elements from Atg3^{ΔNFR} and Atg3^{FL}. **e**, Close-up superposition of Atg3^{ΔNFR} (light blue) and Atg3^{FL} (cyan) structures, showing interactions between Atg3's catalytic domain and E123IR, and conformational rearrangements upon E123IR dislocation.

Figure 3-9. Structural remodeling of the Atg3 catalytic core upon displacement or removal of the E123IR element.

a, Stereo view of Atg3^{ΔNFR} crystal structure in 2Fo-Fc density map, $\sigma=1.0$. **b**, Schematics of structures of Atg3, with E123IR bound (from Atg3^{FL} PDB 2DYT), with E123IR removed in Atg3^{ΔNFR} (this study, PDB 6OJJ), and with E123IR dislodged upon binding to Atg7 (PDB 4GSL), with the conformation in the Atg7-bound complex shown to the right for comparison to the structure with the E123IR deleted shown in **Figure 3-8d**. **c**, Superposition of the structure of Atg3^{ΔNFR} (this study, light blue, PDB 6OJJ) and Atg3^{FL} (cyan PDB 2DYT) shows potential rearrangement of Atg3's extreme C-terminal element between E123IR-bound and -displaced forms of Atg3. Residues not visible in the prior structure are highlighted with spheres for alpha carbons. The conformation of Atg3's extreme C-terminal residues observed in the Atg3^{ΔNFR} structure is incompatible with E123IR binding due to clashing, and is consistent with NMR chemical shift perturbations of this region detected by NMR in **Figure 3-6**.

Table 3-1. Data collection and refinement statistics

Parameters	Atg3^{ΔNFR} (ScAtg3 Δ1-18, Δ86-159)
Data collection	
Space group	C 1 2 1
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	92.078, 44.557, 66.2
α , β , γ (°)	90, 102.977, 90
Resolution (Å)	30-2.40 (2.44-2.40)*
<i>R</i> _{merge}	0.050 (0.766)
<i>I</i> / σ (<i>I</i>)	7.9 (1.1)
<i>CC</i> _{1/2}	0.996 (0.587)
Completeness (%)	92.6 (77.3)
Redundancy	3.4 (2.8)
Refinement	
Resolution (Å)	26.41-2.50
No. reflections	9090
<i>R</i> _{work} / <i>R</i> _{free}	0.2127 / 0.2427
No. atoms	1519
Protein	1458
Water	61
B factors (Å ²)	51.20
Protein	51.24
Water	50.23
R.m.s. deviations	
Bond lengths (Å)	0.006
Bond angles (°)	0.918

*Values in parentheses are for highest-resolution shell.

(Tyr179 and His232) that are important for the lipidation reaction (**Figure 3-8d**, right) (Kaiser et al., 2013; Sakoh-Nakatogawa et al., 2013; Yamada et al., 2007). By contrast, in the absence of the Atg3^{E123IR} element, Atg3's catalytic center is structurally rearranged into an activated conformation through a domino-like effect that ultimately results in remodeling a loop observed in full-length Atg3 into an additional helical turn at the N-terminus of the HR (**Figure 3-8e**); the remodeled helix cannot restrain the side-chain from catalytic Cys234, thereby allowing the alternative orientation where Cys234 is surrounded by additional key catalytic residues (**Figure 3-8d**, left). Moreover, it appears that the extreme C-terminal region of Atg3 also rearranges between the E123IR-bound and other states: this element undergoes a conformational change and additional C-terminal residues are visible in the crystal structure of Atg3^{ΔNFR} relative to the prior structure of full-length Atg3. In the conformation in Atg3^{ΔNFR} these residues would clash with the bound E123IR (**Figure 3-9b,c**), although their nearby location also suggests potential to positively interact with the E123IR in an alternative, presently unknown conformation. Indeed, resonances from these residues display large NMR chemical shift perturbations when ¹⁵N labeled Atg3^{cat} is titrated with the unlabeled FR (**Figure 3-6c**).

Mutants displacing Atg3^{E123IR} from Atg3^{cat} activate ligation

Interestingly, the activated orientation of the catalytic center was observed previously in structures of full length Atg3 when bound to Atg7 (**Figure 3-9b**) (Kaiser et al., 2013; Yamaguchi et al., 2012). Although interpreted as stemming from indirect Atg7 interactions with the backside of Atg3's catalytic domain (Kaiser et al., 2013; Yamaguchi et al., 2012), and/or from the high pH of the crystallization conditions (Sakoh-Nakatogawa et al., 2013), reinterpretation of the crystal structures with the knowledge that the Atg3^{E123IR} element binds Atg3's catalytic domain in solution leads to a prediction: interactions with Atg3^{E123IR} would allosterically restrict Atg3's catalytic domain. However, displacement of the Atg3^{E123IR} element, either by deleting the FR domain (**Figure 3-8d**), or upon binding to the E1 Atg7 during formation of the Atg3~Atg8 intermediate (**Figure 3-9b**), or upon binding to the Atg12–Atg5 portion of E3 for the lipidation reaction (Sakoh-Nakatogawa et al., 2013), would enable the active conformation. Nonetheless, relevance for the lipidation would depend on the Atg3^{E123IR} element binding in the context of an Atg3~Atg8 complex. We tested this concept by NMR. First, a stable proxy for Atg3^{ΔFR}~Atg8 was made with a disulfide bond between a Cys replacement for the C-terminal Atg8 residue and the catalytic Cys in Atg3^{ΔFR} (Atg3^{ΔFR}-S-S-Atg8). Upon adding this to the ¹⁵N-labeled Atg3^{FR}, we observed the similar chemical shift perturbations in [¹⁵N, ¹H] TROSY spectra of ¹⁵N-labeled Atg3^{FR} (**Figure 3-10a**) as for Atg3 alone (**Figure 3-6d**).

If binding to the Atg3^{E123IR} would be inhibitory, then mutations in catalytic domain residues that mediate this interaction and that are not needed for other Atg3 functions should stimulate the lipidation reaction in the absence of E3. Therefore, based on the crystallographic (**Figure 3-8e**) and NMR data (**Figure 3-6f**), we generated several Ala substitutions designed to disrupt the autoinhibitory interactions as follows: Asp133,

Figure 3-10. Mutations in E123IR-binding residues activate Atg3~Atg8 in the absence of E3 *in vitro* and *in vivo*.

a, [^{15}N , ^1H] TROSY spectra of ^{15}N -labeled Atg3^{FR} alone (red) or in 1:2 mixture with unlabeled, disulfide-bonded proxy for Atg3^{ΔFR}~Atg8 (purple), with Chemical Shift Perturbations (CSPs) per residue shown below. **b**, Locations of mutations a-e designed to impair interactions between Atg3's catalytic domain and E123IR shown on crystal structure of Atg3 (PDB 2DYT). **c**, Effects of indicated mutants in Atg3 catalytic domain-E123IR interface on intrinsic E3-independent activity of Atg3~Atg8 intermediate. Quantification is of WT or indicated mutant versions of Atg3~Atg8 remaining after 2.5 minutes as a function of NH_2OH concentration in pulse-chase assays, without E3, repeated 3 times repeats average. (Error bar: STDEV, N = 3) Representative gel is shown in **Figure 3-11**. **d**, Effects of indicated mutants in Atg3 catalytic domain-E123IR interface on E3-independent Atg8 lipidation *in vitro*, in reactions with Atg7, Atg12–Atg5-Atg16, and liposomes generated from *E. coli* polar lipids as a source PE, and detected by migration of Atg8 in Coomassie-stained SDS-PAGE gel. **e**, Effects of indicated mutants in Atg3 catalytic domain-E123IR interface on E3-independent Atg8 lipidation *in vivo*, as detected by western blot for Atg8 after 4 hours starvation of the YCY131 multi-Atg knockout strain of *S. cerevisiae* expressing Atg7, Atg10, Atg8ΔR (activated in absence of Atg4) and either WT or mutant HA-tagged Atg3.

which is the only residue in the Atg3^{E123IR} that does not bind Atg7 and thus could form an Atg3~Atg8 intermediate, interacts with the HR in full-length Atg3; Arg251 and Arg254 in the HR; Lys292, Thr295, and Ser296 in the Atg3^{cat} domain; and Gln302, His303, and Asp304 or Glu308 and Trp310 at the extreme C-terminus (**Figure 3-10b**). These mutations mimicked effects of adding E3 to the *in vitro* reactions lacking E3: relative to wild-type Atg3, they activate the Atg3~Atg8 thioester-bonded intermediate (**Figure 3-10c** and **Figure 3-11**) and the lipidation reaction (**Figure 3-10d**), but are either not affected or defective for E3-dependent activity (**Figure 3-3b** and **Figure 3-12c**). The defect for one mutant in E3-dependent activity can be explained due to its location in the E123IR (D133A), but other mutants do not map to a region presently known to be required for activity, although they may reflect an activated conformation of the Atg3~Atg8 intermediate. If this were the case, then the activation observed in the absence of E3 may even be lower than theoretically possible.

Notably, increased Atg3~Atg8 discharge was not observed upon adding liposomes together with the isolated Atg7^{NTD} that like E3 also binds the Atg3^{E123IR} (**Figure 3-11**). Thus, releasing the Atg3^{E123IR} from the Atg3^{cat} domain may not be sufficient for activating the lipidation activity of the Atg3~Atg8 intermediate. A potential difference between binding of the Atg7^{NTD} versus Atg12~Atg5, or the effects of E3-mimicking mutations, would be if the latter alter internal structural dynamics of the Atg3~Atg8 intermediate so as to increase susceptibility to nucleophilic attack.

To test the effects of the activating mutants *in vivo*, it is necessary to examine Atg8 lipidation in the absence of the proteins comprising the E3. We also wished to minimize confounding effects of Atg8, Atg3, and the Atg12~Atg5~Atg16 complexes interacting with many proteins associated with autophagy and non-autophagy-related pathways. Thus, we generated a minimal system for examining Atg8 lipidation *in vivo*, with a plasmid expressing Atg7, HA-tagged versions of Atg3, and the processed form of Atg8 in a yeast strain in which these and 21 other autophagy (Atg) genes have been deleted (MKO) (Cao et al., 2009). Atg8 lipidation was markedly increased with the majority of the mutants (**Figure 3-10e**), which is remarkable given the complexity of events required for the reaction and the large number of other proteins that interact with Atg3 and Atg8 *in vivo*.

Extensive surfaces are required for Atg3~Atg8 activation

To identify the portions of Atg8 and the Atg3 catalytic domain contributing to the activated state, we performed mutagenesis. E3-dependent discharge was monitored for 28 mutant versions of Atg8 and 27 mutant versions of Atg3, each having one to four Ala replacements for surface residues (**Figure 3-12a,b** and **Figure 3-13a,b**). The Atg8 mutations together probed the majority of its surface, while Atg3 mutations encompass the catalytic domain that is structurally homologous to canonical E2s and the short C-terminal extension. A strikingly large fraction of the mutants affected activation of Atg8 discharge. Notably, the majority of the most defective mutants were also defective for

Figure 3-11. Mutations in interface between Atg3's E123IR element and catalytic domain activate the Atg3~Atg8 intermediate.

Representative gel image of assay from **Figure 3-10c**.

Figure 3-12. Alanine scan within Atg3^{cat} and Atg8 by NH₂OH discharge assays.
a, Representative gel image of assay from **Figure 3-13a**. **b**, Representative gel image of assay from **Figure 3-13b**.

Figure 3-13. Extensive surfaces of Atg8 and the Atg3 catalytic domain are required for activation of the Atg3~Atg8 intermediate.

a, E3-dependent activation of Atg3~Atg8 intermediate testing roles of indicated surfaces through multiple-Ala scanning mutagenesis over Atg3's catalytic domain. Quantification is of WT or indicated mutant versions of Atg3~Atg8 remaining after 2.5 minutes as a function of NH₂OH concentration in pulse-chase assays, with Atg12~Atg5~Atg16 as E3. **b**, E3-dependent activation of Atg3~Atg8 intermediate testing roles of indicated surfaces through multiple-Ala scanning mutagenesis over Atg8, performed as in **a**. **c**, Effects of covalent Atg3 complex formation with Atg8, as detected by comparing ¹⁵N, ¹H TROSY spectra of ¹⁵N-labeled Atg8 G116C (red) alone and disulfide-bonded complex with Atg3^{cat} as a proxy for Atg3^{cat}~Atg8 intermediate (cyan), with chemical shift perturbations per Atg8 residue shown below. Stripes indicate resonances with line-broadening due to intermediate exchange.

Atg8 lipidation *in vitro* (**Figure 3-14a,b**) and *in vivo* (**Figure 3-14c**). For Atg3, the defective mutants map to a large continuous surface encompassing Atg3's α 1-, HR-, and α 5- (large central), α 4- (adjacent to the catalytic center) helices, and C-terminal extension (**Figure 3-15b**). For Atg8, the mutations map to two major surfaces. One corresponds to the canonical UBL hydrophobic patch and C-terminus, and the other to the Atg8-specific groove that binds AIM/LIR sequences in partner proteins (**Figure 3-15c**).

We hypothesized that some of the mutational defects could reflect impaired interactions between Atg8 and Atg3 in the covalent intermediate. Thus, we performed NMR considering that prior studies of intrinsically active E2~UB intermediates showed chemical shift perturbations indicating noncovalent interactions between the covalently linked E2 and UB moieties. Thus, we compared [^{15}N , ^1H] TROSY spectra for ^{15}N -labeled Atg8 harboring a C-terminal Cys alone versus in a disulfide-bonded complex linked to the active site of unlabeled Atg3^{cat} – i.e., in a stable proxy for the Atg3^{cat}~Atg8 intermediate (**Figure 3-13c**).

The striking similarity between the locations of Atg8 NMR resonances shifting upon covalent complex formation with Atg3 (**Figure 3-15c**), and of mutants impairing E3-activated discharge from Atg3 (**Figure 3-15b**) raise the possibility of noncovalent interaction in a so-called closed conformation. We used structural modeling to consider potential parallels between the activated Atg3~Atg8 intermediate and a RING E3-E2~UB complex as representative. First, the structure of Atg3 was superimposed with the homologous region of the E2 UBE2D1 in the intermediate, and then the UB-fold domain of Atg8 was docked onto the linked UB (**Figure 3-15a**). Next, the locations of mutations impairing activity, and NMR chemical shift perturbations in the Atg3^{cat}~Atg8 complex, were analyzed, and found to be in striking concordance with the corresponding interface residues in canonical E2~UB intermediates (**Figure 3-15b,c,d**). The notable exceptions were the AIM/LIR docking site in Atg8, Atg3 residues corresponding to E3-binding regions of canonical E2s, and the extreme C-terminus of Atg3. These regions are not shared between Atg3 and canonical E2s, and the latter region is not fully visible in the crystal structures presumably due to potential to adopt different conformations. Nonetheless, the differences for the autophagy-specific regions that are observed between the crystal structures (**Figure 3-9c**), and in NMR chemical shift perturbations (**Figure 3-6c**), for the E123IR-bound and other forms of the Atg3 catalytic domain are consistent with roles in allosteric regulation. Indeed, in using the Atg3^{ANFR} crystal structure for the structural model, the C-terminal residues of Atg3 approach the docked Atg8. On this basis, it is tempting to speculate that this sequence, Met-Glu-Gly-Trp, would adopt a reverse AIM/LIR motif anchoring Atg8 to Atg3 in a closed conformation. Concordantly, mutation of these residues has amongst the most deleterious effects (**Figure 3-13a** and **Figure 3-12a, 3-14a**), although future studies will be required to visualize the structure of the E3-activated Atg3~Atg8 intermediate.

Figure 3-15. Modeling of the active conformation of Atg3~Atg8 intermediate.

a, Generation of model for a potential closed E2~Ub conformation for Atg3~Atg8, with structures of Atg3^{ΔNFR} (PDB 6OJJ, from this study) and Atg8 (PDB 2ZPN) superimposed on E2 and Ub, respectively, in a RING E3-E2~Ub complex (PDB 4AP4). **b**, Sites of Atg3 mutations impairing E3-dependent activation of Atg3~Atg8, mapped on model for closed conformation. Red – residues corresponding to E2~Ub interface in closed conformation; wheat – residues corresponding to RING E3 binding site; bronze – residues in catalytic segment. **c**, Sites of Atg8 mutations impairing E3-dependent activation of Atg3~Atg8, mapped on model for closed conformation. Red – residues corresponding to E2~Ub interface in closed conformation; orange – residues corresponding to AIM/LIR-binding site. **d**, Atg8 residues showing chemical shift perturbation one standard deviation above the mean upon covalent complex formation with Atg3, mapped on model for closed conformation. Red – residues corresponding to E2~Ub interface in closed conformation; orange – residues corresponding to AIM/LIR-binding site.

Data Availability

The coordinates and structure factors for Atg3^{ΔNFR} have been deposited to the RCSB Protein Data Bank with ID 6OJJ for immediate release upon manuscript publication. The resonance assignments for Atg3^{cat}, Atg3^{FR} (residues 86-159), and Atg8 have been deposited to the Biological Magnetic Resonance Data Bank with the IDs 27922, 27923, and 27924, respectively for immediate release upon manuscript publication.

CHAPTER 4. DISCUSSION

Here we demonstrated that Atg3 is restrained by an E123IR element within its 70-residue flexible region bracing the active site; this brace establishes regulation by interacting with E1 (**Figure 3-6a**) (Taherbhoy et al., 2011), E2 (**Figure 3-6c,d,f**), and E3 (**Figure 3-5a,c**); and that a key function of the autophagy E3 Atg12–Atg5–Atg16 is to bind this brace to activate the intrinsic reactivity of the thioester-bonded Atg3~Atg8 intermediate (**Figure 3-6b**) (Zheng et al., 2019). This unprecedented allosteric mechanism underlying E2 activation - revealed by our NMR, crystallographic, and mutational data, taken together with prior studies - addresses the longstanding question of how Atg8 lipidation is activated by an E3 that lacks sequence and mechanistic conservation with canonical conjugation enzymes. Notably, E1-binding to the E123IR element is not sufficient to activate the Atg3~Atg8 intermediate (**Figure 3-11**), suggesting additional roles for E3 other than binding. Meanwhile, NMR data raise the possibility that Atg3's active site residues undergo conformational motions on a different time-scale from the rest of catalytic domain, which could impact enzymatic activity (**Figure 3-6c**). Taken together, we speculate that E3 binding to the Atg3~Atg8 intermediate broadly influences dynamic or structural features of Atg3's catalytic domain that in turn bias the active site in favor of the reactive conformation. Indeed, the need for E3-binding to activate the Atg3~Atg8 intermediate *in vitro* is partially ameliorated by mutations that map to the surfaces in and adjacent to the Atg3^{cat}-Atg3^{E123IR} interface (**Figure 3-10c,d** and **Figure 3-11**). Physiological relevance is supported by such mutants also activating E3-independent lipidation *in vivo* (**Figure 3-10e**).

Although future studies will be required to visualize downstream steps in the reaction cascade, we propose that Atg8 lipidation involves Atg7, Atg3, and Atg12–Atg5–Atg16 toggling their common interacting region in Atg3 as follows: 1) Prior to encountering the Atg7~Atg8 intermediate, the Atg3^{E123IR} binds to the Atg3 catalytic domain to protect the catalytic Cys from access by non-specific cellular molecules. 2) The Atg7~Atg8 intermediate binds to Atg3's E123IR element using Atg7's N-terminal domain. This mediates both E1-E2 interactions and conformational activation of Atg3's catalytic Cys to receive Atg8 from Atg7. 3) Following formation of the Atg3~Atg8 intermediate, Atg3's E123IR element could re-engage the Atg3 catalytic domain to prevent Atg8 mis-ligation to a non-specific nucleophile. We speculate that this may also prevent the extreme C-terminal residues of Atg3 from prematurely adopting a conformation that contributes to the activate conformation of the Atg3~Atg8 intermediate. 4) With relatively higher binding affinity, the Atg12–Atg5 portion of the autophagy E3 subsequently dislodges Atg3's E123IR, which enables structural remodeling of the active site loop from a sequestered position to an exposed conformation where the catalytic Cys is juxtaposed with surrounding side-chains that are crucial for Atg8 lipidation (**Figure 4-1**). Apparently, Atg12–Atg5 further activates the lipidation reaction in a manner involving extensive surfaces from both Atg3 and Atg8 in the Atg3~Atg8 intermediate. In parallel with the mechanisms how RING and SUMO E3 activate canonical E2~UBL intermediates (Buetow and Huang, 2016; Cappadocia and Lima, 2018; Zheng and Shabek, 2017), we speculate that the autophagy E3

Figure 4-1. Schematic model for allosteric regulation of Atg3 activity through E123IR interactions across the lipidation cascade.

The autophagy E2 Atg3 is autoinhibited by Atg^{E123IR}. When Atg3 encounters E1 Atg7, E123IR is relocated upon binding to Atg7^{NTD}, thereby triggering rearrangement of the Atg3 catalytic core to activate Cys234 for attacking the Atg7~Atg8 intermediate. Atg8 is transferred from Atg7 to Atg8, producing the thioester-bonded Atg3~Atg8 intermediate. Relieved from Atg7, Atg3^{E123IR} protects the Atg3~Atg8 intermediate from wayward discharge to errant nucleophiles. The E3 (Atg12–Atg5–Atg16) binds E123IR and further activates the Atg3~Atg8 intermediate for nucleophilic attack. Numerous interactions with membranes place this complex in proximity to PE for the lipidation reaction.

Atg12–Atg5 mediates activation in part by promoting interactions between Atg3 and Atg8 in the Atg3~Atg8 intermediate, although details of the interaction remain elusive and might be distinct for the autophagy enzymes.

Why might the UBL conjugation pathway in autophagy involve such elaborate allosteric control? It seems likely that key features of the ultimate target, and intrinsic capabilities of the Atg3~Atg8 intermediate, impose distinctive requirements for regulation. First, the substrate PE is among the most abundant core glycerophospholipids. Found in several organelles, PE's widespread accessibility differs from canonical UB and UBL substrates, which typically only are licensed for modification after a regulatory event such as a post-translational modification directs E3 binding. Second, through its N-terminal membrane-curvature sensing helix, Atg3 itself can bind the substrate PE (Nath et al., 2014). The active site of Atg3~Atg8 may require protection to prevent inopportune discharge to PE, and this is best ensured by coming from the intermediate itself. Indeed, when conformationally activated by E3-mimicking mutations, Atg3~Atg8 can not only catalyze the lipidation reaction and *in vivo*, but also robustly discharges Atg8 to nonspecific nucleophiles even in the absence of E3. Thus, our data present a mechanism ensuring that Atg8 discharge is coupled to E3 binding, which in turn establishes targeting specificity through several features, including binding to cargo receptors (Fracchiolla et al., 2016), and to membranes (Lystad et al., 2019; Romanov et al., 2012). The Atg16 moiety in the autophagy E3 could subsequently engage the lipidated Atg8 to serve as a scaffold during autophagosome biogenesis (Kaufmann et al., 2014).

Allosteric E2 activation is emerging as a common property of E3 ligases (Buetow and Huang, 2016; Cappadocia and Lima, 2018; Zheng and Shabek, 2017). It is now well-recognized that many E3 catalytic domains stabilize a “closed” conformation that renders E2~UB and E2~UBL intermediates susceptible to nucleophilic attack, while other E3 domains mediate substrate targeting. Many E2s are further activated allosterically, through their backside beta-sheets binding to noncatalytic E3 domains. These effects have largely been characterized for indirectly increasing binding to E3 RING domains. However, some E3 or E3-associated domains also trigger conformational changes at E2 active sites. For example, the membrane E3-associated Cue1p binds to Ubc7p's backside, and indirectly modulates the active site, tens of angstroms away (Metzger et al., 2013). Irrespective of whether E2 cysteines require protection from errant nucleophilic attack, or coordination of catalytic activation with other E3 functions, it seems likely that allosteric modulation of E2 active sites serves as a failsafe ensuring UB and UBL discharge at appropriate targets. The autophagy E2 Atg3 now serves as an ultimate case, with its brace self-imposed until directly lifted by the E3 to drive autophagy.

Apparently, lifting off the E123IR from Atg3 in the Atg3~Atg8 intermediate is not sufficient to fully activate Atg8-lipid conjugation, since adding E3 to WT Atg3 accelerates Atg8 lipidation more robustly than the auto-releasing Atg3 mutants in absence of E3. Moreover, our preliminary data from introducing additional E123IR peptides into *in vitro* constituted Atg8 lipidation system shows that E123IR is not sufficient to fully block the reaction. Here we suspect that other elements within the Atg3 FR may also help tuning the interactions between Atg3~Atg8 intermediate and Atg12–

Atg5-Atg16. Our hypothesis can be tested by other research methods including solving the structure of the active conformation of Atg3~Atg8-Atg12-Atg5-Atg16 complex. However, since Atg3~Atg8 adopts multiple conformations shifting between active and inactive states, obtaining homogeneous *in vitro* recombinant protein samples of Atg3~Atg8-Atg12-Atg5-Atg16 can be exigent. Besides, since Atg3 contains several long flexible regions (e.g. FR and partial HR) that couldn't be removed due to their indispensable roles in enzyme function, it will be very challenging crystallizing the full complex. It is also potentially taxing to obtain cryo EM structures to display details of all the functional parts if they are largely unstructured.

As we discovered in this study, the C-terminus of Atg3 is associated with Atg8 when Atg3~Atg8 intermediate adopts the closed conformation, and its distinctive reversed AIM-like amino acid sequence of Met-Glu-Gly-Trp leads to the speculation that such interaction is realized by Atg8-AIM binding. In fact, interactions between Atg8 family protein and C-terminal LIR/AIM of other autophagic enzymes are not unrepresented. The C-terminus of the autophagy E1 Atg7 docks into the AIM-binding groove of its substrate Atg8, though it doesn't harbor a traditional AIM sequence (Noda et al., 2011). In human protein studies, the Phe-Glu-Ile-Leu sequence approaching the very end of ATG4 serves as a canonical LIR as binding to GABARAPL1, and was proved essential for ATG4's protease activity (Skytte Rasmussen et al., 2017). Intriguingly, such binding event similar to that between Atg8 and Atg3 C-terminus also takes place between canonical E2 and UBL. For example, the last few amino acids of yeast Cdc34 was proved directly binding to its substrate ubiquitin. Such interaction is functionally pronounced for Cdc34's catalytic activity (Choi et al., 2010).

According to our preliminary result of the Atg8 lipidation assay conducted in *S. cerevisiae in vivo* cells, not all alanine mutagenesis introduced to the Atg3-binding sites in Atg8 showed decreased densities in lipidated Atg8 bands, while some mutants even harbor significantly higher lipidation levels. As we speculate, this inconsistency against the *in vitro* results using the same set of mutants (**Figure 3-1**) could be due to counteracting impacts from Atg4 against Atg3: for some Atg8 mutants, PE conjugation activity is impaired by defective Atg3-binding; however Atg4-mediated Atg8 cleavage from autophagic membrane is also diminished at the same time. Intriguingly and as expected, the Atg3- and Atg4-binding surfaces are largely overlapped on Atg8 if using human LC3-ATG4B structure for comparison (Satoo et al., 2009). The results bring up a reasonable hypothesis that Atg8 binding to Atg3 and Atg4 is mutually exclusive, given Atg4 and Atg3 potentially play opposite roles in maintaining the total amount of lipidated Atg8 during autophagy progression. The unknown molecular requirements determining Atg8's preference between Atg4 and Atg3 indicate that Atg8-PE conjugation, as well as the whole autophagy flux, involves more complicated and delicate signaling pathways that need to be further studied.

Researchers now have limited knowledge about the membrane binding functions within enzymes in Atg8 lipidation cascade. Human protein study has shown that the N-terminal helix of Atg3 serves as membrane curvature detector which prefers binding to higher curvature liposome (Nath et al., 2014). Similar to other canonical E3 ligases that

create physical proximity between E2~UBL intermediates and their specific substrates by binding to both, Atg12–Atg5–Atg16 also harbors membrane binding affinity in order to bring Atg3~Atg8 to substrate PE. According to the study in *Saccharomyces Cerevisiae*, membrane binding is mediated by Atg5, activated by Atg16, but inhibited by Atg12 (Romanov et al., 2012). Tested with in vitro lipid vesicles, this membrane binding feature enables the E3 complex to tether isolated membrane in absence of Atg8, and is also essential for autophagosome engulfment more than just for Atg8 lipidation (Romanov et al., 2012). However, the Atg5-lipid binding event takes place later than Atg12–Atg5–Atg16 being located to the pre-autophagosomal structure (PAS) (Romanov et al., 2012), which suggests that the membrane system also utilizes other E3 recruiting mechanism besides Atg5 interaction. In addition, recent studies reported multiple lipid-recognition domains across ATG16L1, including its N-terminal amphipathic helix, the C-terminal end, and the coiled-coil domain (Lystad et al., 2019). Notably, ATG16L1 harbors intrinsic lipid-binding that is independent from WIPI-mediated recruitment to PAS (Lystad et al., 2019). Multiple binding sites within the E2 and E3 complex, especially when they do not rely on one another or other lipid-binding machineries, indicates sophisticated regulatory mechanism orchestrating Atg8 family protein lipidation. The complicated nature of Atg12–Atg5–Atg16 interaction to PAS also hypothesizes that the roles of Atg12–Atg5–Atg16 in autophagy may be of greater variety than only to directly facilitate Atg8 transfer from Atg3 to PE.

According to current studies, inhibitors for autophagic proteins, especially for enzymes in Atg8 family protein lipidation cascade, are not being extensively investigated. Potential drug screenings against the lipidation enzymes may provide therapeutics to related diseases. Considering this, a great variety of distinctive mechanisms in the lipidation machinery can be targeted. Obviously, making protein-protein interaction inhibitors is a reasonable idea to directly suppress LC3 lipidation activity, and to consequentially downregulate the entire autophagy level. Besides, cysteine interaction inhibitors could specifically attenuate the nucleophilic attacking efficiencies of autophagic E1 or E2, yet not interrupting E2-UBL, E2-substrate and E3-E2 interactions. Nonetheless, our study also sheds light on the possibility to design Atg3 allosteric inhibitors, which allows more fine-tuned regulation by arresting Atg8 family protein lipidation in inactive states, and avoids disturbing any intrinsic enzyme activities. In summary, the dynamic nature of the Atg8 family protein lipidation system has now become an opportunity for drug discovery.

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