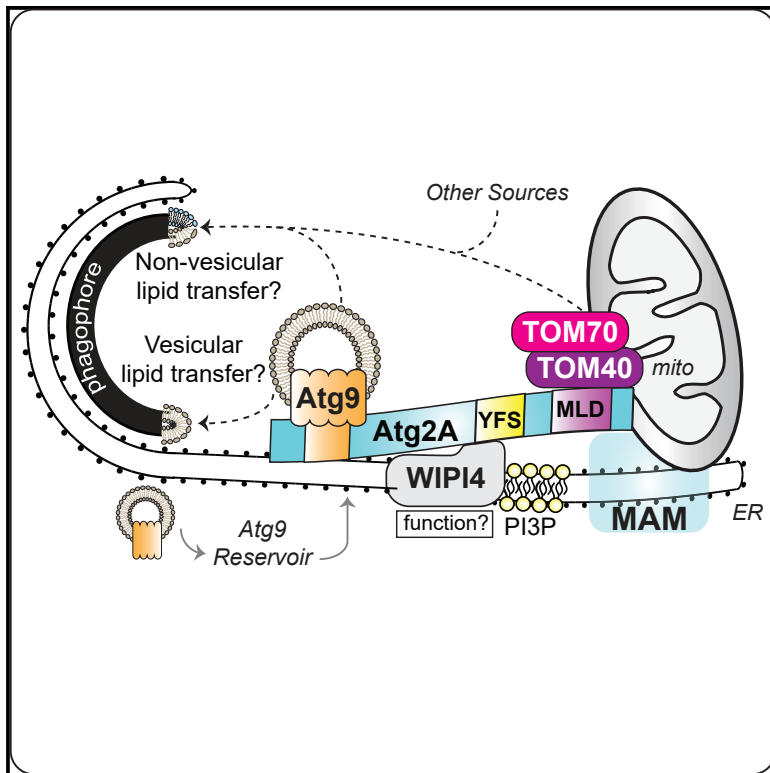


# TOM40 Targets Atg2 to Mitochondria-Associated ER Membranes for Phagophore Expansion

## Graphical Abstract



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## In Brief

Tang et al. show that human Atg2 is a key regulator for phagophore expansion. TOM40/70 directs Atg2A to MAM to mediate phagophore expansion. On the MAM, Atg2A facilitates Atg9-vesicle delivery and retrograde trafficking to promote phagophore expansion and efficient autophagic flux.

## Highlights

- Human Atg2 functions at the phagophore expansion step
- Atg2A translocates to MAM during autophagy
- TOM40/70 complex recruits Atg2A to MAM
- Atg2A regulates Atg9-vesicle delivery for phagophore expansion at MAM



# TOM40 Targets Atg2 to Mitochondria-Associated ER Membranes for Phagophore Expansion

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<https://doi.org/10.1016/j.celrep.2019.07.036>

## SUMMARY

During autophagy, phagophores grow into double-membrane vesicles called autophagosomes, but the underlying mechanism remains unclear. Here, we show a critical role of Atg2A in phagophore expansion. Atg2A translocates to the phagophore at the mitochondria-associated ER membrane (MAM) through a C-terminal 45-amino acid domain that we have termed the MAM localization domain (MLD). Proteomic analysis identifies the outer mitochondrial membrane protein TOM40 as a MLD-interacting partner. The Atg2A-TOM40 interaction is responsible for MAM localization of Atg2A and requires the TOM receptor protein TOM70. In addition, Atg2A interacts with Atg9A by a region within its N terminus. Inhibition of either Atg2A-TOM40 or Atg2A-Atg9A interactions impairs phagophore expansion and accumulates Atg9A-vesicles in the vicinity of autophagic structures. Collectively, we propose a model that the TOM70-TOM40 complex recruits Atg2A to the MAM for vesicular and/or non-vesicular lipid transport into the expanding phagophore to grow the size of autophagosomes for efficient autophagic flux.

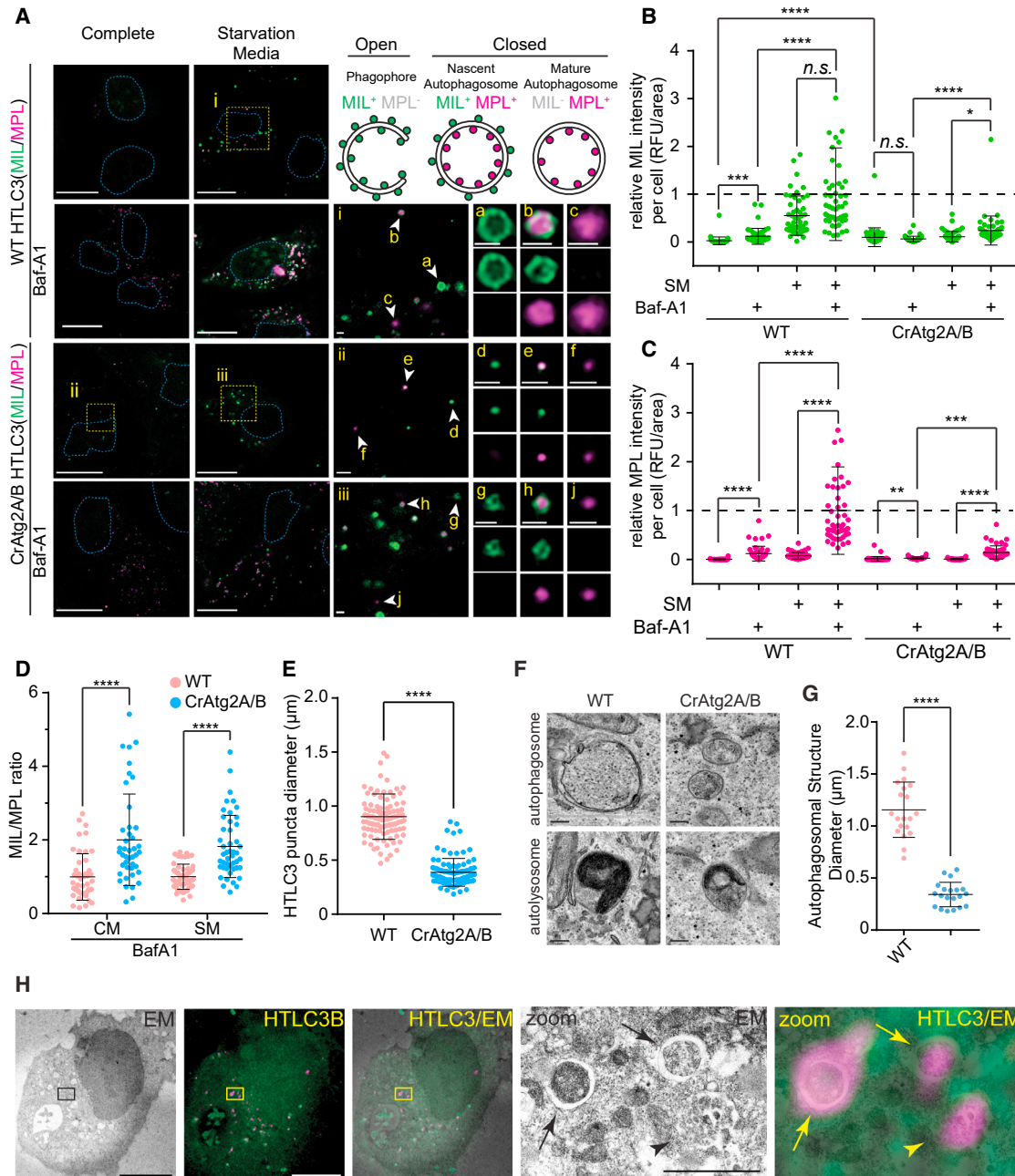
## INTRODUCTION

Macroautophagy (hereafter autophagy) is an evolutionarily conserved intracellular degradation process that plays a vital role in the maintenance of cellular and tissue homeostasis (Mizushima et al., 2008). Dysregulation of autophagy has been implicated in the pathogenesis of various human diseases, including cancer, neurodegenerative disease, and vascular disease (Choi et al., 2013). Moreover, at the cellular level, autophagosomal membranes have been reported to function as signaling platforms for cell death and survival, including apoptosis, necroptosis, and mitogen-activated protein (MAP)-kinase pathways (Goodall et al., 2016; Martinez-Lopez et al., 2013; Tang et al., 2017). Thus, understanding the molecular mechanism of autophagosome biogenesis is pivotal for the development of future autophagy-targeting therapeutics.

The process of autophagy begins with the formation of the phagophore, which undergoes membrane expansion and eventually seals to form a double-membrane vesicle, known as the autophagosome (Mizushima et al., 2011). The autophagosome is then fused with endosomes and lysosomes to deliver the sequestered materials for hydrolytic degradation and nutrient recycling. Although the molecular mechanism of autophagosome biogenesis remains far from clear, yeast genetic screens have identified over 30 autophagy-related (ATG) genes that are important for autophagy (Klionsky, 2004; Nakatogawa et al., 2009). Several upstream ATG proteins, including components of the class III phosphatidylinositol 3-kinase complex, translocate to the mitochondria-associated endoplasmic reticulum (ER) membrane (MAM) for autophagosome formation (Hamasaki et al., 2013). Among these ATG proteins, the Atg8/LC3 family of proteins is one of the most studied molecules involved in autophagosome formation. During autophagy, cytosolic LC3-I is conjugated to the lipid phosphatidylethanolamine (PE) to generate LC3-II on the phagophore. The LC3-PE conjugation occurs during phagophore expansion and serves to recruit ubiquitinated cargo through direct interaction with cargo adaptors, such as p62/sequestosome-1. Upon phagophore closure, LC3-II on the outer autophagosomal membrane (OAM) is delipidated and recycled to the cytosol prior to lysosomal fusion, while LC3-II on the inner autophagosomal membrane (IAM) is degraded upon autophagosome-lysosome fusion (Kabeya et al., 2000; Mizushima et al., 2001; Tanida et al., 2005). Therefore, LC3-II serves as a widely used marker for autophagy (Klionsky et al., 2016).

Although the lipidation of LC3 is associated with phagophore expansion, the mechanism and membrane source for such process remains unknown. Atg9 is the sole transmembrane ATG protein that is required for autophagosome formation (Webber et al., 2007). Although Atg9 basally resides in the *trans*-Golgi network (TGN) and recycling endosomes known as the Atg9 reservoir, vesicles containing Atg9 bud off from the reservoir in response to autophagic stimuli and traffic to the autophagosome formation site (Orsi et al., 2012; Takahashi et al., 2011; Takahashi et al., 2016; Yamamoto et al., 2012). Here, Atg9 only transiently associates with autophagosomal membranes and is rapidly recycled back to the reservoir for reuse (Orsi et al., 2012; Webber et al., 2007). As Atg9 shuttling between the reservoir and phagophore is important for controlling the number and size of autophagosomes (Imai et al., 2016; Yamamoto et al., 2012), Atg9





**Figure 1. Atg2A/B Is Required for Autophagosomal Membrane Expansion**

(A) HaloTag-LC3 (HT-LC3)-expressing wild-type (WT) and Atg2A/B double knockout (CrAtg2A/B) U-2 OS cells were incubated in complete media (CM) or starvation media (SM) in the presence or absence of 100 nM Baf-A1 for 2 h and subjected to the HT-LC3 autophagosome completion assay followed by confocal microscopy. Magnified images of the boxed (i–iii) and arrow-indicated areas (a–j) are shown in the middle and right panels, respectively. Schematic illustration of phagophore (MIL<sup>+</sup>MPL<sup>-</sup>), nascent autophagosome (MIL<sup>+</sup>MPL<sup>+</sup>), and mature autophagosome (MIL<sup>-</sup>MPL<sup>+</sup>) are shown on the upper right panel. Scale bars represent 20  $\mu\text{m}$  and 1  $\mu\text{m}$  in the magnified images.

(B and C) The cytoplasmic fluorescence intensities of MIL (B) and MPL (C) in each cell under indicated conditions were quantified and normalized to the respective mean fluorescence intensities of WT cells starved in the presence of Baf-A1 ( $n = 50$ ).

(D) MIL/MPL ratio for each cell incubated in CM or SM in the presence of Baf-A1 was calculated and normalized to the mean of fluorescence intensities of WT cells ( $n = 50$ ).

(E) The diameters of HT-LC3 foci randomly selected from the starved WT and CrAtg2A/B were quantified using the Velocity software ( $n = 100$ ).

(F) WT and CrAtg2A/B U-2 OS cells were starved for 2 h and subjected for TEM. Representative autophagosome-like and autolysosome-like structures are shown on the upper and lower panels, respectively. Scale bars represent 200 nm.

(G) The diameter of each autophagic structure randomly selected from the electron micrographs was quantified ( $n = 20$ ).

(legend continued on next page)

vesicles are proposed to deliver membrane and other components for phagophore expansion.

In yeast, Atg2 interacts with Atg9 to promote autophagy and regulate Atg9 retrieval from the pre-autophagosomal structure (PAS) (Feng et al., 2014; Gómez-Sánchez et al., 2018). Atg2 is one of the least understood ATG proteins required for autophagy (Gómez-Sánchez et al., 2018; Suzuki et al., 2013; Velikkakath et al., 2012). Previous studies in yeast have shown that Atg2 can be recruited to PAS through the interaction with the phosphatidylinositol 3-phosphate (PI3P)-binding protein Atg18 (Obara et al., 2008; Suzuki et al., 2007). In mammalian cells, two Atg2 orthologs (Atg2A/B) and four Atg18 orthologs (WIPI1–4) have been identified (Proikas-Cezanne et al., 2015; Velikkakath et al., 2012). Although Atg2A and Atg2B are functionally redundant to promote autophagic degradation (Velikkakath et al., 2012), WIPI proteins appear to be functionally non-redundant (Proikas-Cezanne et al., 2015). Among WIPIs, WIPI4 exhibits the strongest physical interaction with mammalian Atg2 and has been reported to be involved in omegasome maturation and autophagosome formation (Lu et al., 2011; Zheng et al., 2017). Although the physiological significance of mammalian Atg2-WIPI4 interaction remains unknown, the loss of Atg2 accumulates cytoplasmic punctate structures containing Atg9/Atg9A, Atg1/ULK1, Atg14, and Atg16/Atg16L1 in both yeast and human cells (Tang et al., 2017; Velikkakath et al., 2012), suggesting the importance of Atg2 proteins in autophagosome biogenesis.

Here, we demonstrate that the loss of mammalian Atg2A/B significantly impairs autophagy at the phagophore expansion step. During autophagy, Atg2A translocates to the autophagosome formation site at the ER-mitochondria contact site by a C-terminal MAM localization domain (MLD). Although inhibition of Atg2A recruitment to the MAM impairs phagophore expansion and autophagic flux, the MAM targeting of Atg2A occurs independent of its binding to WIPI4 but requires the TOM70-TOM40 complex. Furthermore, we demonstrate that Atg2A directly interacts with Atg9A at the MAM to promote phagophore growth. These results implicate mammalian Atg2A as a critical regulator of Atg9A vesicle delivery and phagophore expansion during autophagosome biogenesis at the MAM.

## RESULTS

### Atg2 Is Required for Autophagosomal Membrane Expansion

Mammalian Atg2A/B proteins are functionally redundant and have been reported to be indispensable for basal and starvation-induced autophagic flux in THP-1 and HeLa cells (Tang et al., 2017; Velikkakath et al., 2012). Consistently, we found that the loss of Atg2A/B impaired lysosomal turnover of LC3-II and p62 in U-2 OS cells under both basal and starved conditions (Figures S1A and S1B). To clarify the role of Atg2A/B in autophago-

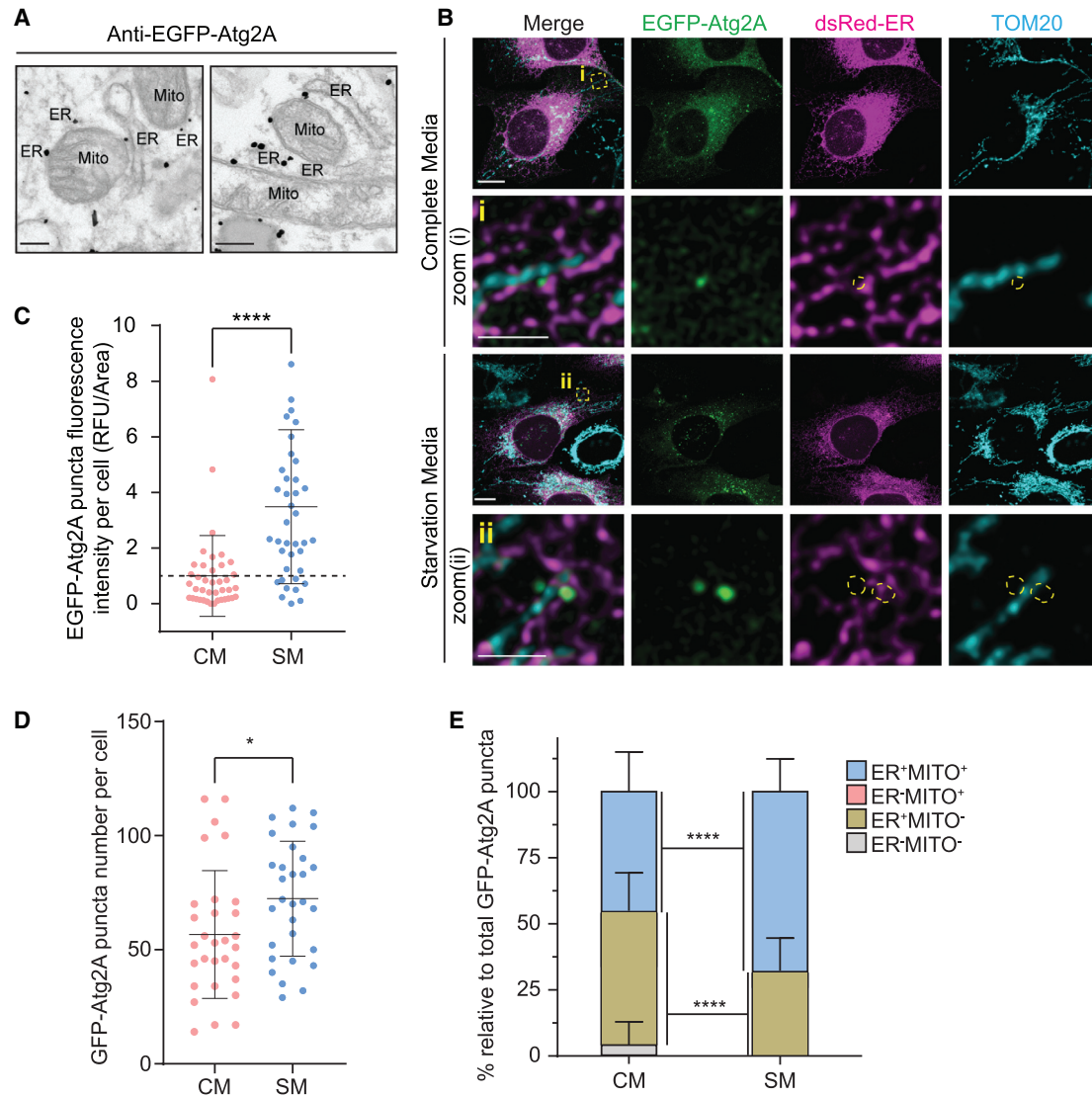
some biogenesis, we next took the advantage of the HaloTag-LC3 (HT-LC3) autophagosome completion assay. Briefly, this assay is able to distinguish expanding phagophores from nascent autophagosomes and mature autophagosomes or autolysosomes by sequentially labeling cytosol-accessible and autophagosome-sequestered HT-LC3 by using fluorescently tagged membrane-impermeable (MIL) and membrane-permeable (MPL) HaloTag ligands, respectively (Takahashi et al., 2018). As expected, phagophores (MIL<sup>+</sup>MPL<sup>-</sup>), autophagosomes (MIL<sup>+</sup>MPL<sup>+</sup>), and mature autophagic structures (MIL<sup>-</sup>MPL<sup>+</sup>) appeared in the cytoplasmic region of starved wild-type (WT) cells (Figures 1A a–c). Interestingly, all three autophagic structures (MIL<sup>+</sup>MPL<sup>-</sup>, MIL<sup>+</sup>MPL<sup>+</sup>, and MIL<sup>-</sup>MPL<sup>+</sup> HT-LC3 foci) were also detected in Atg2A/B double knockout (CrAtg2A/B) cells (Figure 1A, d–j). Moreover, the addition of the lysosomal V-ATPase inhibitor Bafilomycin A1 (Baf-A1) slightly but significantly increased MPL signals in CrAtg2A/B cells (Figure 1C), indicating that autophagosomes still can form and mature into autolysosomes in the absence of Atg2A/B. Strikingly, however, we found that the MIL/MPL ratio in the presence of Baf-A1 in CrAtg2A/B cells was significantly higher than that in WT cells to indicate the accumulation of phagophores and/or autophagosomes (Figure 1D). Moreover, MIL signals were significantly increased in CrAtg2A/B cells even under non-starved conditions (Figures 1B), suggesting that Atg2A/B loss delays autophagosome completion under both basal and induced conditions.

Notably, the sizes of HT-LC3 puncta in CrAtg2A/B U-2 OS cells were significantly smaller than those in WT cells (Figure 1E). To verify this phenotype, we performed transmission electron microscopy (TEM). Consistently, although autophagosome- and autolysosome-like structures were observed in both WT and CrAtg2A/B cells (Figure 1F), these autophagic structures were significantly smaller in Atg2A/B-deficient cells (Figure 1G). Taken together, these results indicate that mammalian Atg2A/B functions at the membrane expansion step during autophagosome biogenesis.

It has recently been reported that Atg2A/B deficiency in HeLa cells fails to form typical phagophores and autophagosomes and results in the accumulation of LC3 signals on small, single-membrane vesicle-like structures (Tamura et al., 2017). We, thus, next performed correlative light and electron microscopy (CLEM) and confirmed that HT-LC3-positive foci in CrAtg2A/B U-2 OS cells indeed represented autophagosome- and autolysosome-like structures (Figure 1H). To determine if the discrepancy is due to the difference in cell types, we generated Atg2A/B-deficient HeLa and HEK293T cells (Figures S1C and S1G) and analyzed autophagic structures by the HT-LC3 assay and electron microscopy. Consistent with the phenotype observed in U-2 OS cells, small autophagic structures were detected in Atg2A/B-deficient HEK293T and HeLa cells (Figures S2C–S2I), further supporting the role of mammalian Atg2A/B in phagophore expansion rather than formation.

(H) HT-LC3-expressing CrAtg2A/B U-2 OS cells were starved for 2 h, stained for with MIL and MPL, and subjected to CLEM. Magnified images of the boxed areas are shown on the right panels.

Arrows and arrowheads indicate autophagosome-like and autolysosome-like structures, respectively. Scale bars represent 10  $\mu$ m and 1  $\mu$ m in the magnified image. Statistical significance was determined by one-way ANOVA followed by Dunn's multiple comparisons test (B and C) or t test (D, E, and G). All values are mean  $\pm$  SD; n.s., not significant; \* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ ; \*\*\*\* $p \leq 0.0001$ .



**Figure 2. Atg2A Localizes to MAM upon Autophagy Induction**

(A) CrAtg2A/B U-2 OS cells were transduced with lentiviruses encoding sgRNA-resistant EGFP-Atg2A, starved for 2 h and subjected to immunoelectron microscopy using anti-GFP antibody. Scale bars represent 200 nm.

(B) EGFP-Atg2A- and dsRed-ER-expressing CrAtg2A/B U-2 OS cells were incubated in CM or SM for 2 h, stained for TOM20, and analyzed by confocal microscopy. Magnified images of the boxed areas are shown in the lower panels. Scale bars represent 10  $\mu$ m and 2.5  $\mu$ m in the magnified images.

(C) The fluorescence intensity of EGFP-Atg2A in each cell under indicated conditions was quantified and normalized to the respective mean fluorescence intensity of cells incubated in CM (n = 40).

(D and E) Total EGFP-Atg2A puncta number per cell (D) and the percentage of EGFP-Atg2A puncta localize to cytosol (ER<sup>-</sup>MITO<sup>-</sup>), ER (ER<sup>+</sup>MITO<sup>-</sup>), mitochondria (ER<sup>-</sup>MITO<sup>+</sup>), or MAM (ER<sup>+</sup>MITO<sup>+</sup>) under CM or SM were quantified (n = 30) (E).

Statistical significance was determined by t test (C and D) or one-way ANOVA followed by Dunn's multiple comparisons test (E). All values are mean  $\pm$  SD. \*p  $\leq$  0.05; \*\*\*\*p  $\leq$  0.0001.

### Atg2A Localizes to ER-Mitochondria Contact Sites

To understand the mechanism behind the regulation of phagophore expansion by Atg2, we first determined the precise intracellular location of Atg2 by immunoelectron microscopy by using CrAtg2A/B cells stably expressing single guide RNA (sgRNA)-resistant EGFP-Atg2A. Interestingly, upon nutrient starvation, EGFP-Atg2A signals were detected at ER-mitochondria contact sites, also known as the MAM (Figure 2A). The localization of

Atg2A was further determined by confocal microscopy. Consistent with a previous report (Velikkakath et al., 2012), EGFP-Atg2A signals were detected throughout the cytoplasmic region as well as in small foci structures under fed conditions (Figure 2B). The fluorescence intensity as well as the number of EGFP-Atg2A puncta was significantly increased by starvation (Figures 2B–2D), suggesting the translocation of Atg2A during autophagy. Notably, although nearly all Atg2A puncta were found

to be associated with the ER regardless of the nutrient status, a portion of the ER-associated Atg2A signals was also positive for a mitochondrial marker under fed condition (Figures 2B and 2E). However, the percentage of Atg2A puncta associated with both ER and mitochondrial markers was significantly increased by starvation (Figures 2B and 2E), indicating the accumulation of Atg2A at MAM during autophagy.

Most recently, the membrane-tethering function of Atg2 has been demonstrated *in vitro* using liposomes (Neubert et al., 2019; Osawa et al., 2019; Valverde et al., 2019). Because Atg2A translocates to the MAM during starvation, we next examined if Atg2A/B is important for the formation of ER-mitochondrial contact sites. Electron microscopy showed that the number of MAM in CrAtg2A/B cells was comparable to that in WT cells (Figures S2A and S2B), indicating that the reduction of autophagosomal membrane size by Atg2A/B depletion is not due to the impairment of MAM formation.

### Atg2A-WIPI4 Interaction Is Dispensable for Atg2A Function in Phagophore Expansion

It has been reported that knock down of Atg2A/B accumulates GFP-WIPI1-positive LC3 foci (Velikkakath et al., 2012). Consistently, we observed an accumulation of the PI3P marker mRFP-FYVE on GFP-ULK1-positive early autophagic structures in CrAtg2A/B cells in a manner that is dependent on the activation of the PI3-kinase (Figures S2C–S2E). Because the PI3P-effector Atg18 is required for the PAS recruitment of Atg2 in yeast (Obara et al., 2008), we next examined if WIPI4, a mammalian Atg18 ortholog, is crucial for Atg2A MAM localization and the function of Atg2A in phagophore expansion. Instead of WIPI4 knockout, which will disrupt multiple cellular functions of WIPI4, we used an Atg2A(YFS-AAA) mutant that abolishes Atg2A-WIPI4 interaction (Figures S3A and S3B), as previously reported (Zheng et al., 2017). To our surprise, the mutations in the YFS motif did not block the starvation-induced colocalization of EGFP-Atg2A with dsRed-ER and TOM20 (Figure S3C). Moreover, similar to EGFP-Atg2A(WT), EGFP-Atg2A(YFS-AAA) rescued the defects in phagophore expansion and autophagic degradation (Figures S3D–S3G) in CrAtg2A/B cells. Collectively, these results indicate that the Atg2A-WIPI4 interaction is dispensable for Atg2A translocation to the MAM and the phagophore expansion function of Atg2A.

### TOM40 Is an Interacting Partner of Atg2A That Binds to the MLD

To determine the minimal region of Atg2A that is required for MAM localization, we next generated a series of Atg2 deletion mutants (Figure S4A). We found that a 45-amino acid sequence near the C-terminal end of Atg2A (amino acid 1776–1820) and located within the previously characterized autophagosome and lipid droplet localization region (Velikkakath et al., 2012) is sufficient for Atg2A localization to ER-mitochondria contacting sites and, thus, named this region the MLD. Notably, expression of EGFP-Atg2A(1776–1820) (EGFP-MLD) in CrAtg2A/B cells results in MAM localization regardless of nutrient status (Figures S4B and S4C).

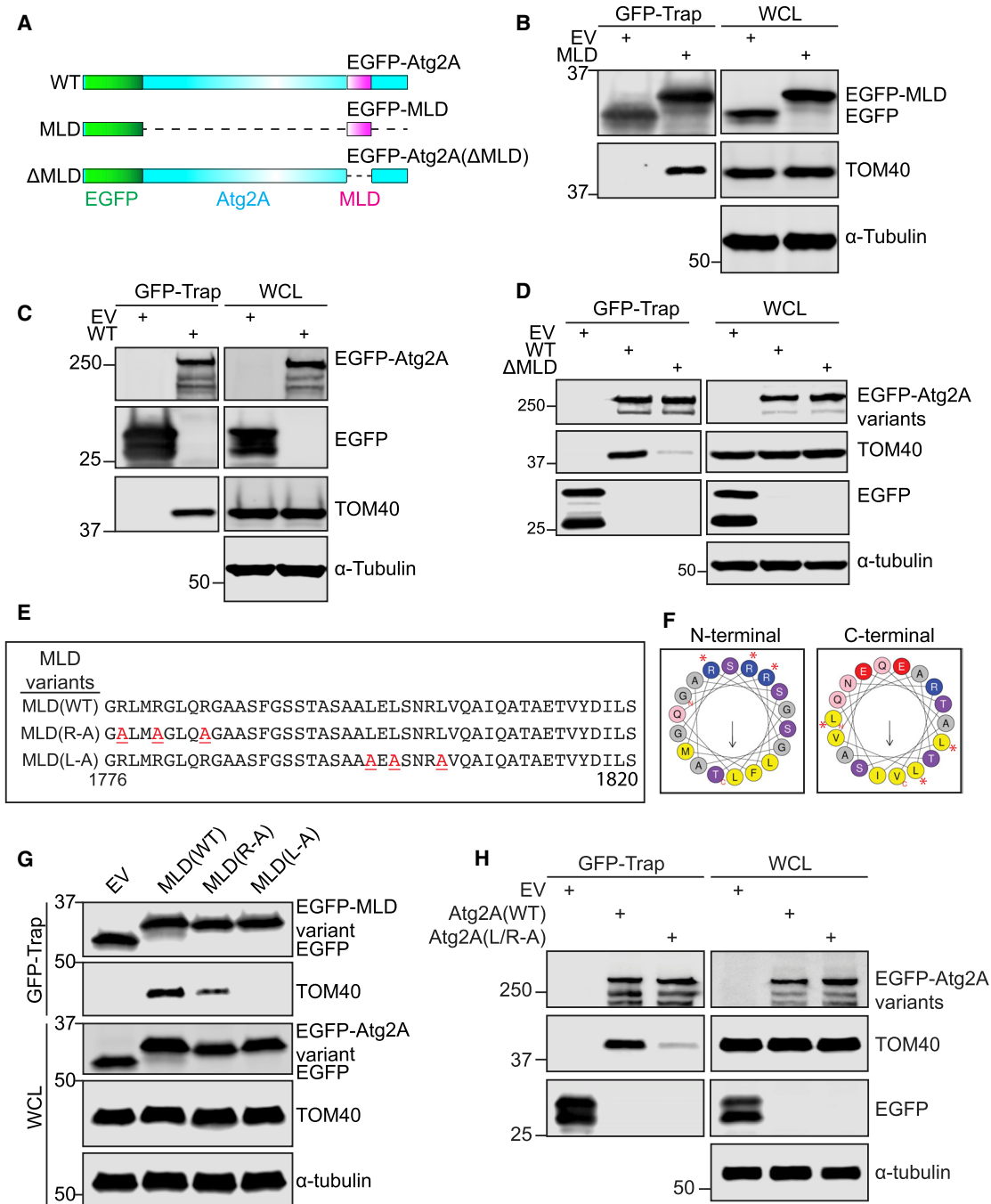
To understand the mechanism underlying the MAM localization of Atg2A, we next took a proteomic approach to identify pro-

teins that associate with the MLD. As Atg2A was previously suggested to only transiently associate with LC3-positive foci (Velikkakath et al., 2012), we took the advantage of photo-amino acid UV-crosslinking coupled with affinity purification and proteomics. To minimize the contamination of non-labeled bait proteins and the induction of apoptosis that is observed upon prolonged overexpression of EGFP-MLD (Figures S4D and S4E), we generated doxycycline-inducible EGFP-empty or EGFP-MLD-expressing CrAtg2A/B cells. The resultant cells were incubated with doxycycline together with photo-leucine and photo-methionine followed by UV-crosslinking, GFP-Trap, and mass spectrometry. We recovered a total of 750 preys, and by employing EGFP-empty as a normalization control and a 5-fold increase threshold, 75 potential MLD interacting partners were identified and categorized according to their subcellular localization (Table S1). Consistent with the MAM localization of the MLD, over 55% of the potential interacting partners are either mitochondria- or ER-related proteins (Table S1), and these were selected for further analyses.

After validation by co-immunoprecipitation in the absence of photo-amino acid labeling and UV photo-crosslinking, we identified TOM40, the central component of the translocase of the outer mitochondrial membrane (TOM) complex (Dekker et al., 1998), as a MLD-binding protein (Figures 3A and 3B). The interaction of TOM40 with full-length Atg2A was confirmed by co-immunoprecipitation using anti-TOM40 antibodies or ectopic expression of EGFP-Atg2A followed by GFP-Trap (Figures S5A and 3C). To confirm that MLD mediates the interaction between full-length Atg2A and TOM40, we generated an EGFP-Atg2A mutant lacking the MLD (Figure 3A) and examined its interaction with TOM40. As expected, TOM40 interaction was abolished in the absence of MLD (Figure 3D), indicating that Atg2A interacts with TOM40 through the MLD. Importantly, the interaction of Atg2A with TOM40 was enhanced by starvation (Figures S4A and S4B), suggesting a role of TOM40 in the MAM translocation of Atg2A during autophagy. In contrast, the depletion of upstream ATGs including Atg13 and Atg9A had little effect on the interaction of Atg2A with TOM40 (Figures S5C and S5D). Collectively, these results identify TOM40 as an interacting partner of Atg2A that binds by its N-terminal MLD.

### MLD Harbors Two Putative $\alpha$ Helices That Are Essential for TOM40 Interaction

To determine the residues of the MLD that are responsible for binding to TOM40, we first aligned the human Atg2A MLD protein sequence with its orthologs. We found that the MLD region and especially several positively charged and hydrophobic residues within this domain are highly conserved among all higher eukaryotes analyzed (Figure S6A). We next predicted the secondary and tertiary structure of the human MLD of Atg2A (Figures S6B and S6C) (Xu and Zhang, 2012, 2013). We found that the 45 amino acids of MLD comprise two  $\alpha$  helices that are linked by a short coil (Figure S6C). Notably, three highly conserved arginine residues in the N-terminal helix of MLD are oriented on the same side (Figures 3E, 3F, and S6C), suggesting that these residues may mediate membrane binding (Li et al., 2013; Schwieger and Blume, 2009). Indeed,



**Figure 3. The MLD of Atg2A Is Responsible for the Interaction with TOM40**

(A) Schematic illustration of EGFP-Atg2A variants.

(B–D, G, and H) CrAtg2A/B HEK293T cells were transfected with the indicated EGFP-Atg2A variants for 24 h and subjected to immunoprecipitation with GFP-Trap beads followed by immunoblotting with the indicated antibodies. EV and WCL represent EGFP-empty vector and whole-cell lysate, respectively.

(E) Amino acid sequences of the MLD and its variants used in the study are shown. Red underlined characters indicate the point mutations introduced into the sequences.

(F) Helical wheels of N-terminal (left) and C-terminal (right)  $\alpha$  helices of MLD were plotted using HeliQuest. Red asterisks indicate amino acid residues substituted to alanine.

we observed that the arginine substitution to alanine (Figure 3F) reduced the foci formation of the MLD (Figure S6D). We next determined if the arginine residues are also important for the interaction with TOM40. We found that the MLD and TOM40 interaction was greatly suppressed by the arginine to alanine mutations (Figure 3G), suggesting that the arginine residues on the N-terminal helix of MLD are important not only for membrane recognition but also for the interaction of Atg2A with TOM40.

Photo-leucine and photo-methionine are used for the photo-amino acid UV-crosslinking assay because protein-protein interactions frequently require the adaptation of hydrophobic residues into hydrophobic pockets of its interacting partner, such as p21-PCNA and BH3-mediated interactions of Bcl-2 family proteins (Gulbis et al., 1996; Shamas-Din et al., 2013). To determine if the conserved hydrophobic leucine residues on the C-terminal helix of MLD (Figure S6A) are required for interaction with TOM40, we generated a MLD mutant harboring leucine to alanine mutations (Figures 3E and 3F). Notably, both foci formation (Figure S6D) and TOM40 interaction (Figure 3G) were abolished by the leucine to alanine substitutions in the MLD. To confirm the importance of both helical regions of the MLD in the interaction of Atg2A with TOM40, we generated a full-length Atg2A mutant harboring both arginine and leucine to alanine substitutions in the MLD. Consistently, mutation of the conserved residues abolished the interaction of Atg2A with TOM40 (Figure 3H). Notably, Atg2A(L/R-A) maintains its interaction with WIPI4, whereas the WIPI4 interaction-defective Atg2A(YFS-AAA) mutant interacts with TOM40, indicating that the WIPI4 and TOM40 interactions of Atg2A are achieved by two independent domains (Figure S6E). Taken together, these data indicate that the MLD of Atg2A harbors two putative  $\alpha$  helices that are essential for TOM40 interaction.

### The MAM Localization of Atg2A Is Required for Phagophore Expansion and Autophagic Flux

To investigate whether TOM40 interaction of Atg2A is required for its MAM localization, we first analyzed the intracellular localization of the TOM40 interaction-defective Atg2A( $\Delta$ MLD) and Atg2A(L/R-A) mutants. We found that neither EGFP-Atg2A( $\Delta$ MLD) nor EGFP-Atg2A(L/R-A) formed puncta or localized to MAM in response to nutrient starvation (Figures 4A and 4B), suggesting the importance of the Atg2A-TOM40 interaction in the membrane localization of Atg2A. We next investigated whether the MAM localization of Atg2A is crucial for the expansion of phagophores. In contrast to EGFP-Atg2A(WT), neither EGFP-Atg2A(L/R-A) nor EGFP-Atg2A( $\Delta$ MLD) rescued the phagophore expansion defect in Atg2A/B-deficient cells (Figures 4C and 4D), indicating that MAM localization is a prerequisite for Atg2A to exert its biological function for proper membrane expansion. Furthermore, only EGFP-Atg2A(WT), but not EGFP-Atg2A(L/R-A) nor EGFP-Atg2A( $\Delta$ MLD), restored the impaired autophagic flux in CrAtg2A/B cells (Figures 4E–4J). Taken together, these data indicate that Atg2A exerts pro-autophagic function at the ER-mitochondria contacting sites by promoting phagophore expansion for efficient autophagic degradation.

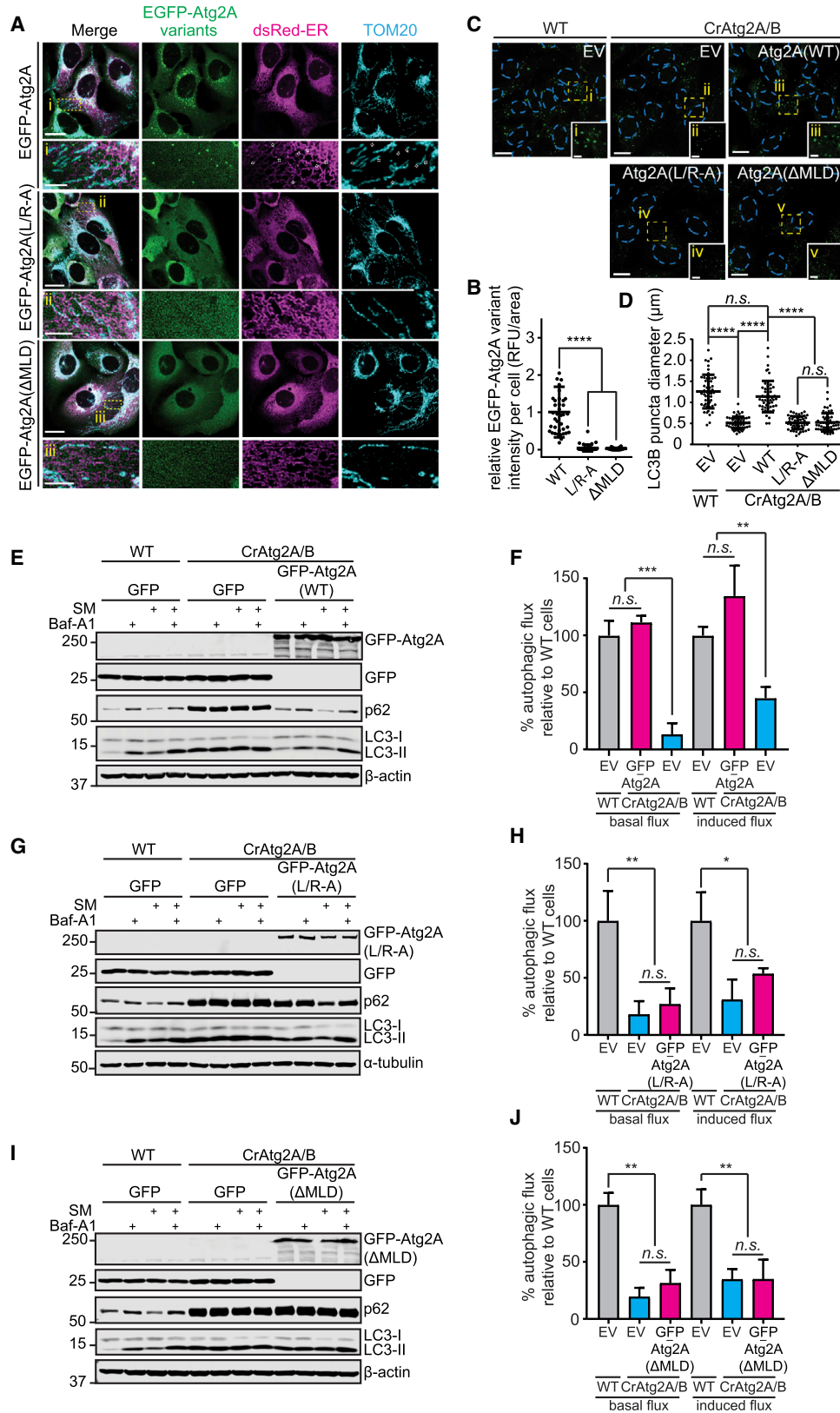
### TOM70 Mediates the Atg2A-TOM40 Interaction for Phagophore Expansion

Several additional TOM components, including TOM70, were detected in our proteomics analysis but failed to reach the five-fold enrichment threshold (Figure 5A). TOM70 and TOM20 are two of the major TOM receptors that transiently associate with the core TOM complex to deliver targeted proteins to TOM40 (Rapaport, 2002). Interestingly, a recent study has shown that TOM70, but not TOM20, clusters at MAM to recruit inositol trisphosphate receptor calcium transfer (Filadi et al., 2018). To determine if the TOM70-dependent delivery process is required for the Atg2A-TOM40 complex formation, TOM70-deficient U-2 OS cells were generated (Figure 5B) and subjected to co-immunoprecipitation analysis. Our data showed that the interaction of Atg2A with TOM40 was impaired by the loss of TOM70 (Figure 5C), suggesting a role of the TOM70 pathway in the recruitment of Atg2A to MAM during autophagy. To examine the importance of TOM70-dependent recruitment of Atg2A in phagophore expansion, we used the rapamycin-induced FKBP (FK506-binding protein)/FRB (FKBP-rapamycin binding domain) dimerization system (Inobe and Nukina, 2016). To artificially induce the recruitment of Atg2A to MAM and the outer mitochondrial membrane (OMM) or the OMM alone, FRB-GFP-Atg2A( $\Delta$ MLD) was co-transduced to CrAtg2A/B cells with TOM70-mRFP-FKBP or TOM20-mRFP-FKBP, respectively. Rapamycin-induced dimerization of FRB-GFP-Atg2A( $\Delta$ MLD) and TOM70-mRFP-FKBP, but not TOM20-mRFP-FKBP, restored the size of LC3 puncta in CrAtg2A/B cells (Figures 5D and 5E), indicating that TOM70-dependent recruitment of Atg2A to MAM is sufficient to induce phagophore growth upon the induction of autophagy. Notably, however, dimerization of FRB-GFP-Atg2A( $\Delta$ MLD) and TOM70-mRFP-FKBP occasionally generated abnormally enlarged LC3-positive structures (Figures 5E), and autophagic flux remained impaired in these cells (Figure 5F). These observations suggest that the dissociation of Atg2A from MAM may be required for functional autolysosome formation.

### Atg2A Interacts with Atg9A and Promotes Atg9A Vesicle Delivery for Phagophore Expansion

In yeast, Atg2 has been shown to interact with Atg9 to promote autophagy (Gómez-Sánchez et al., 2018). Consistently, we observed the interaction between Atg2A and Atg9A in mammalian cells (Figures 6A and S7B). Because Atg9 vesicles have been suggested to be a membrane source for phagophore growth (Webber et al., 2007), we next examined whether mammalian Atg9A is required for proper phagophore expansion. Similar to the loss of Atg2A/B, autophagic flux is impaired and LC3B puncta diameter is significantly reduced in Atg9A-deficient cells (Figures 6B–6D), supporting the importance of Atg9 vesicles in phagophore expansion. We next determined if mammalian Atg2 proteins are involved in Atg9A trafficking during autophagy. Consistent with previous studies (Orsi et al., 2012; Takahashi et al., 2011), Atg9A signals in starved WT cells were dispersed throughout the cytoplasmic region with only a small portion of the signals colocalizing with LC3 (Figures 6E and 6F). In contrast, the loss of Atg2A/B resulted in the accumulation of Atg9A signals on LC3-positive structures (Figure 6E). Using CLEM,





(legend on next page)

we confirmed that the LC3 and Atg9A-positive structures in CrAtg2A/B cells represent autophagic structures (Figure 6G). Interestingly, the autophagic structures in CrAtg2A/B cells were surrounded by 30- to 50-nm Atg9A-GFP-positive vesicles (Figure 6G). Collectively, these results suggest that mammalian Atg2 proteins are dispensable for the anterograde trafficking of Atg9A but may mediate Atg9A vesicle delivery for phagophore expansion.

To better understand the function of mammalian Atg2 proteins at MAM, we next examined if the Atg2-Atg9 interaction is crucial for phagophore expansion and autophagic degradation. Using a series of deletion mutants (Figure S7A), we identified that amino acids 237–431 in the N terminus of Atg2A are essential for the interaction of Atg2A with Atg9A (Figures 7A, 7B, and S7B). As both EGFP-Atg2A and EGFP-Atg2A( $\Delta$ 237-431) harbor intact MLD, similar TOM40 interactions were detected (Figure S7B), suggesting that the Atg2A-Atg9A interaction is achieved independently from the MLD-mediated TOM40 interaction. Consistently, the starvation-induced MAM localization of EGFP-Atg2A( $\Delta$ 237-431) remains unaffected (Figure S7C). Taken together, these data indicate that EGFP-Atg2A( $\Delta$ 237-431) localizes to MAM through interaction with TOM40 in response to autophagic stimuli; however, its interaction with Atg9A is abolished.

We next examined whether the Atg2A-Atg9A interaction at the MAM is required for the phagophore expansion functions of Atg2A. Although both Atg2A(WT) and Atg2A( $\Delta$ 237-431) localize to MAM upon nutrient starvation (Figure S7C), the defects in phagophore expansion (Figures 7C and 7D) and autophagic degradation (Figures 7E and 7F) in CrAtg2A/B cells were only rescued by the restoration of EGFP-Atg2A(WT) but not the EGFP-Atg2A( $\Delta$ 237-431) mutant. Moreover, unlike full-length Atg2A, which restored the defective Atg9A trafficking in CrAtg2A/B cells, the accumulation of Atg9A puncta adjacent to LC3-positive foci remained upon the expression of EGFP-Atg2A( $\Delta$ 237-431) (Figure S7D), indicating the importance of the Atg2A-Atg9A interaction in Atg9A vesicle delivery and subsequent retrograde trafficking. Collectively, these data indicate that Atg2A recruited to the MAM by a C-terminal MLD domain interacts with Atg9A at its N terminus to regulate phagophore expansion.

## DISCUSSION

It has been proposed that the phagophore forms and grows at ER-mitochondria contact sites (Hamasaki et al., 2013), but the

underlying molecular mechanisms have not been fully elucidated. In this study, we demonstrate the importance of the MAM localization of Atg2A in phagophore expansion. We found that the starvation-induced MAM localization of Atg2A is mediated through the interaction of OMM protein TOM70 and TOM40 and that Atg2A-MAM localization is required for proper Atg9A trafficking and autophagosomal membrane expansion. These results provide a mechanistic insight into phagophore growth and further support the importance of MAM in autophagosome biogenesis.

The observation that Atg2A/B depletion accumulates small immature autophagosomal membranes is consistent with previous reports (Tang et al., 2017; Velikkakath et al., 2012). However, although these studies initially suggested a role of Atg2A/B in phagophore closure, our data show that small phagophores in CrAtg2A/B cells still can undergo membrane closure to form autophagosomes that mature into autolysosomes. Because the size of autophagic structures in CrAtg2A/B cells is much smaller than that in WT cells, we propose that the failure of membrane expansion limits efficient membrane closure to accumulate immature autophagosomal membranes. Importantly, the observations that Atg2A/B deficiency significantly reduces the lysosomal turnover of LC3-II and impairs p62 degradation are consistent with the conclusion that Atg2 is required for efficient autophagic flux. Most recently, the involvement of endosomal sorting complex required for transport-III (ESCRT-III)-mediated membrane fission in phagophore closure has been demonstrated (Takahashi et al., 2018). Interestingly, although the sizes of phagophores were minimally affected by the depletion of ESCRT-III CHMP2A, Atg2A accumulated on p62- and Atg9-positive immature autophagic structures in CHMP2A-depleted cells (Takahashi et al., 2018). As the mechanism leading to ESCRT-III recruitment to phagophores is unknown, it will be of interest to determine whether Atg2A-mediated membrane expansion is a prerequisite for efficient recruitment of the ESCRT machinery.

During autophagosome biogenesis, many ATG proteins, including ATG14, DFCP1, ATG16, Beclin1, and VPS34, translocate to the autophagosome formation sites at MAMs (Hamasaki et al., 2013). In this study, we find that Atg2 also accumulates at MAMs during starvation. Interestingly, the MAM localization of Atg2 occurs independent of its ability to bind WIPI4, as the WIPI4 interaction-defective Atg2(YFS-AAA) mutant localizes to the MAM and rescues the defects in phagophore expansion and autophagic degradation in CrAtg2A/B cells. These results are unexpected, as yeast Atg18 has been suggested to dictate the PAS localization of Atg2 through the binding of PI3P

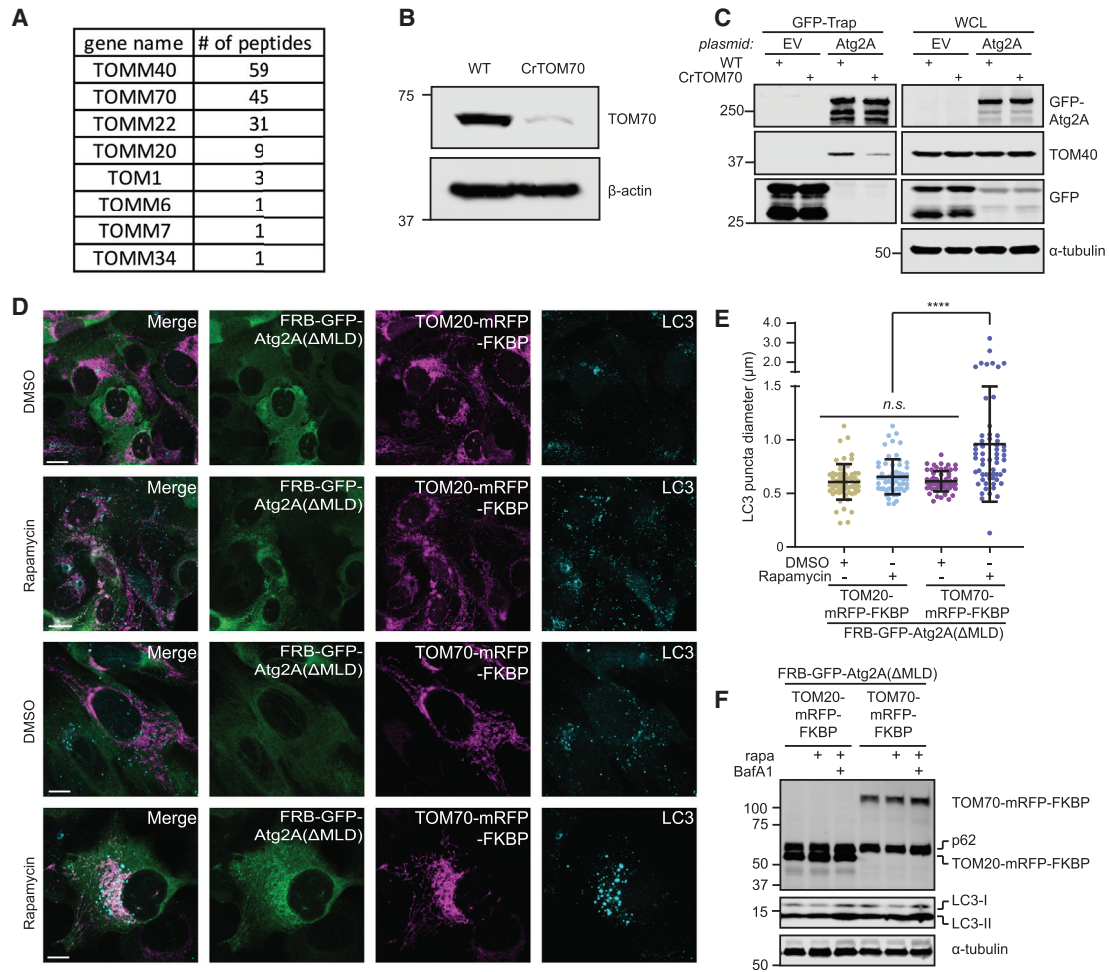
### Figure 4. MAM Localization of Atg2A Is Required for Its Biological Function in Autophagy

(A) CrAtg2A/B U-2 OS cells stably expressing the indicated EGFP-Atg2A variants and dsRed-ER were starved for 2 h, stained for TOM20, and analyzed by confocal microscopy. Magnified images in the boxed areas are shown in the lower panels. Scale bars represent 20  $\mu$ m and 5  $\mu$ m in the magnified images.

(B) The fluorescence intensity in each cell expressing the indicated Atg2A variant was quantified and normalized to the respective mean fluorescence intensity of EGFP-Atg2A(WT) cells (n = 38).

(C and D) U-2 OS cells expressing the indicated EGFP-Atg2A variants were starved for 2 h, stained for LC3, and analyzed by confocal microscopy. In (C), LC3 is pseudo-colored green for visualization, and magnified images in the boxed areas are shown in the insets. Scale bars represent 20  $\mu$ m and 5  $\mu$ m in the insets. EV represents EGFP-empty vector. In (D), the diameters of LC3 foci were randomly selected and quantified using the Velocity software (n = 50).

(E–J) The indicated U-2 OS stable transductants were incubated in CM or SM for 2 h in the presence or absence of 100 nM Baf-A1 and subjected to immunoblotting with the indicated antibodies (E, G, and I). Basal and starvation-induced autophagic flux was quantified (F, H, and J; n = 3) as described in the STAR Methods. Statistical significance was determined by one-way ANOVA followed by Dunn's multiple comparisons test. All values are mean  $\pm$  SD; n.s., not significant; \*p  $\leq$  0.05; \*\*p  $\leq$  0.01; \*\*\*p  $\leq$  0.001; \*\*\*\*p  $\leq$  0.0001.



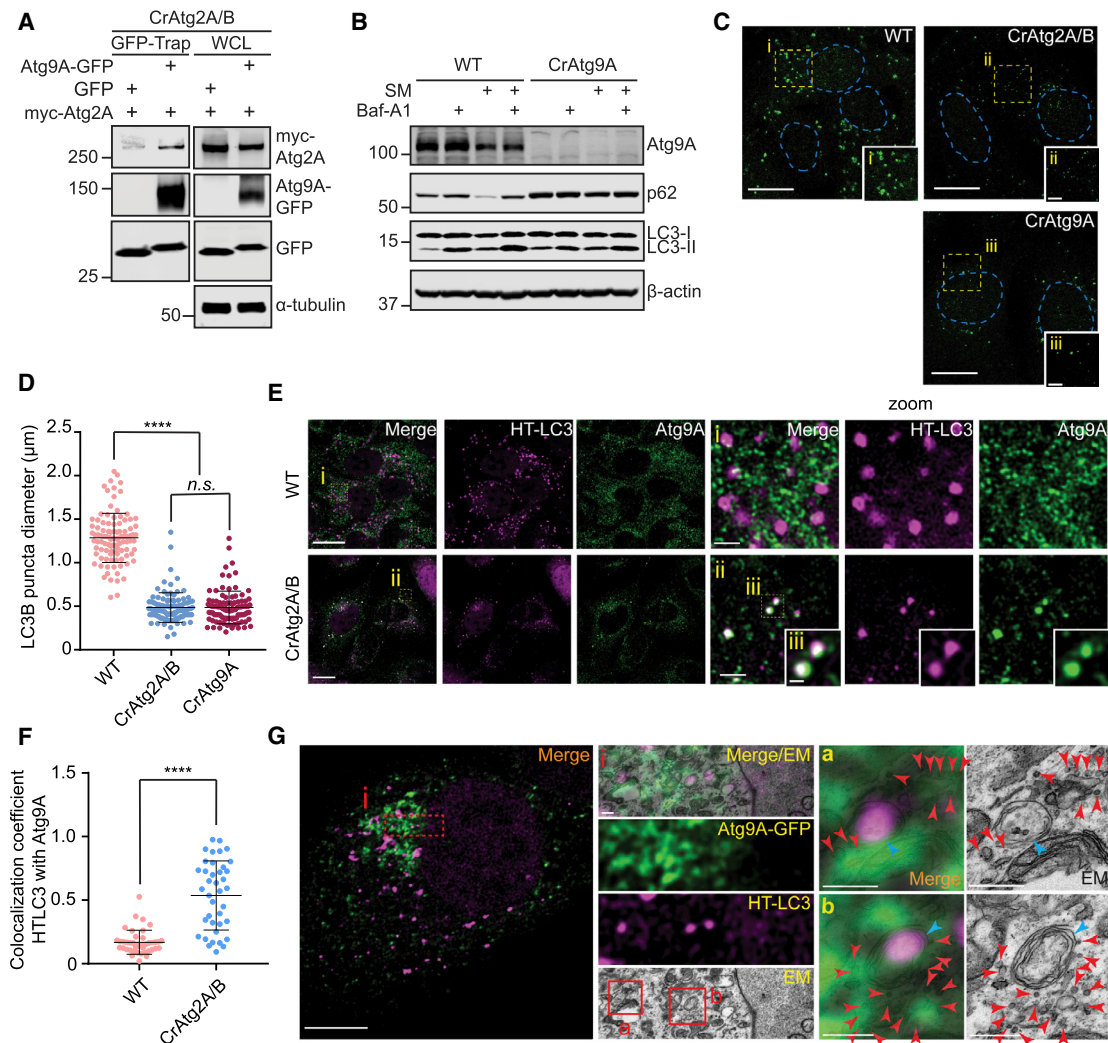
**Figure 5. TOM70 Mediates the Atg2A-TOM40 Interaction and Atg2A-Dependent Phagophore Growth**

(A) TOM components and their corresponding peptide numbers identified in the proteomic analysis using EGFP-MLD are listed. (B) HEK293T cells were transduced with lentiviruses encoding Cas9 and sgRNAs targeting TOM70 (CrTOM70) and subjected to immunoblotting with the indicated antibodies. (C) WT or CrTOM70 HEK293T cells were transfected with the EGFP (EV) or EGFP-Atg2A (Atg2A) for 24 h and subjected to immunoprecipitation with GFP-Trap beads followed by immunoblotting with the indicated antibodies. (D–F) The indicated CrAtg2A/B U-2 OS stable transductants were treated with DMSO or 150 nM rapamycin in the presence or absence of 100 nM Bafilomycin A1 for 8 h and subjected to LC3 immunostaining and confocal microscopy (D) or immunoblotting with the indicated antibodies (F). In (E), the diameters of LC3 foci in (D) were randomly selected and quantified using the Velocity software ( $n = 60$ ). Statistical significance was determined by one-way ANOVA followed by Dunn's multiple comparisons test. All values are mean  $\pm$  SD; n.s., not significant; \*\*\*\* $p \leq 0.0001$ .

(Kobayashi et al., 2012; Obara et al., 2008). Instead, we find that Atg2A localizes to MAM through a 45-amino acid domain located within the previously characterized autophagosome and lipid droplet localization region at the C terminus (Velikkakath et al., 2012). Notably, this domain is highly conserved and contains several essential arginine and leucine residues. As it is the minimal domain required for MAM localization and localizes to the MAM regardless of nutrient status, we named this the MLD.

Our proteomic analysis identifies the TOM component TOM40 as a MLD-interacting protein, and we have shown that TOM40 is responsible for the MAM localization of Atg2A through the TOM receptor TOM70. Despite restored phagophore expansion

in CrAtg2A/B cells upon the artificial targeting of Atg2A( $\Delta$ MLD) to MAM by rapamycin-induced dimerization with TOM70, abnormally enlarged autophagic membranes can be observed and the defect in autophagic flux persists. As immature autophagic structures are reported to be detached from the ER in Atg2A/B-depleted cells (Kishi-Itakura et al., 2014), the regulated assembly and disassembly of Atg2A-TOM40 interactions may be responsible for the establishment of phagophore-MAM contacting sites during phagophore expansion and the proper release of autophagosomes, but this remains to be studied. It also remains to be characterized how the MAM localization of Atg2 is controlled by nutrient status. Unlike full-length Atg2A, EGFP-MLD strongly accumulates at MAM even under nutrient-rich



**Figure 6. Atg2A/B Deficiency Accumulates Atg9 Vesicles at the Autophagosome Formation Sites in the Close Proximity of the MAM**

(A) GFP- or Atg9A-GFP-expressing CrAtg2A/B HEK293T cells were transfected with myc-Atg2A for 24 h and subjected to immunoprecipitation with GFP-Trap beads followed by immunoblotting with the indicated antibodies.

(B) WT and CrAtg9A U-2 OS cells were incubated in CM or SM in the presence or absence of 100 nM Baf-A1 and subjected to immunoblotting with the indicated antibodies.

(C) The indicated U-2 OS cells were starved for 2 h, stained for LC3, and analyzed by confocal microscopy. Magnified images in the boxed areas are shown in the insets. Scale bars represent 20  $\mu$ m and 5  $\mu$ m in the magnified images.

(D) The diameters of LC3 foci were randomly selected and quantified using the Velocity software (n = 100).

(E) HT-LC3-expressing WT and CrAtg2A/B U-2 OS cells were starved for 2 h, stained for Atg9A and MPL (TMR), and analyzed by confocal microscopy. Magnified images of the boxed areas are shown in the right panels (i and ii) and insets (iii). Scale bars represent 20  $\mu$ m, 2  $\mu$ m in the magnified images, and 0.5  $\mu$ m in the insets.

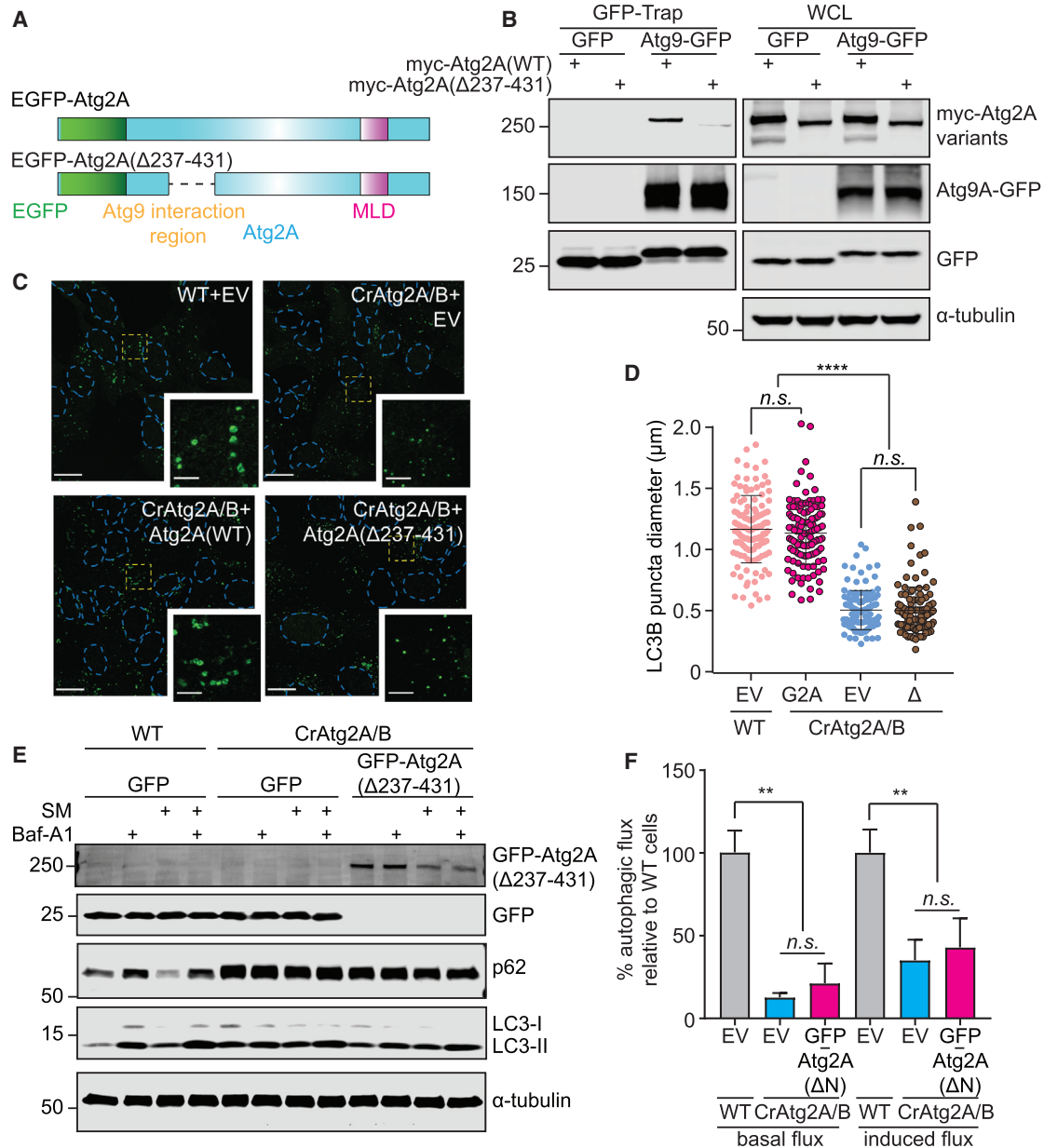
(F) Colocalization coefficient of HT-LC3 with Atg9A per cell was quantified using the Velocity software and shown (n = 40).

(G) CrAtg2A/B U-2 OS cells stably expressing HT-LC3 and Atg9A-GFP were starved for 2 h, stained for MPL (TMR), and subjected to CLEM. Scale bars represent 10  $\mu$ m and 0.5  $\mu$ m in the magnified images. Magnified images of the boxed areas are shown in the middle (i) and right panels (a and b). Red and blue arrowheads indicate Atg9A-GFP-positive vesicles and HT-LC3-positive autophagic structures, respectively.

culture conditions, suggesting that Atg2A may need to undergo conformational change or interact with an unknown co-factor in order to achieve MAM localization in response to autophagy induction.

During the revision of this manuscript, *in vitro* membrane tethering and lipid transfer activities have been demonstrated to reside within the N-terminal region of Atg2 (Neubert et al., 2019; Osawa et al., 2019; Valverde et al., 2019). As mitochondria

have been shown to supply lipids including PE for the biogenesis of autophagosomes (Hailey et al., 2010), it is of future interest to investigate if Atg2A also mediates non-vesicular lipid transport at the MAM for phagophore expansion. Furthermore, the membrane source for the formation and/or expansion of the phagophore may also be supplied by vesicular trafficking (Longatti and Tooze, 2009). In this study, we observed that Atg2A/B deficiency accumulates Atg9A signals around LC3-positive



**Figure 7. The Interaction of Atg2A with Atg9 Is Indispensable for Phagophore Expansion**

(A) Schematic diagrams of EGFP-tagged WT and Atg9A binding-defective Atg2A variant.

(B) GFP- or Atg9A-GFP-expressing CrAtg2A/B HEK293T cells were transfected with myc-Atg2A variants for 24 h and subjected to immunoprecipitation with GFP-Trap beads followed by immunoblotting with the indicated antibodies.

(C) U-2 OS cells expressing the indicated EGFP-Atg2A variants were starved for 2 h, stained for LC3, and analyzed by confocal microscopy. LC3 is pseudo-colored green for visualization, and magnified images in the boxed areas are shown in the insets. Scale bars represent 20 μm and 5 μm in the magnified images. EV represents EGFP-empty vector.

(D) The diameters of LC3 foci were randomly selected and quantified using the Velocity software (n = 120). EV, WT, and Δ represent EGFP-empty vector, EGFP-Atg2A(WT), and EGFP-Atg2A(Δ237-431), respectively.

(E and F) The indicated U-2 OS cells were incubated in CM or SM for 2 h in the presence or absence of 100 nM Baf-A1 and subjected to immunoblotting with the indicated antibodies (E). Basal and starvation-induced autophagic flux in the indicated U-2 OS stable transductants were quantified as described in the STAR Methods (n = 3) (F).

Statistical significance was determined by one-way ANOVA followed by Dunn's multiple comparisons test. All values are mean ± SD; n.s., not significant; \*\*p ≤ 0.01; \*\*\*\*p ≤ 0.0001.

autophagic structures. Although this observation is consistent with the notion that Atg2 regulates Atg9 retrieval from the PAS in yeast (Feng et al., 2014), we find that Atg9A vesicle-like structures are accumulated around autophagic structures at MAM in CrAtg2A/B cells to suggest that the membranes fail to be delivered to the expanding phagophore. Moreover, the Atg9-binding-defective Atg2A( $\Delta$ 237–431) mutant is capable of localizing to the MAM upon nutrient starvation but cannot restore phagophore expansion in CrAtg2A/B cells; thus, Atg2 at the MAM may function to tether or properly orient Atg9-containing vesicles for lipid delivery during phagophore expansion.

Collectively, we identify a minimal 45-amino acid MLD domain within the C terminus of Atg2A that is required for the TOM70/TOM40-mediated translocation of Atg2A to the autophagosome formation site at the MAM for phagophore expansion. Although we show that an N-terminal domain of Atg2A supports Atg9-mediated vesicular lipid transport for efficient autophagic flux, additional membrane tethering functions and/or lipid transfer activities of Atg2A at the MAM is an intriguing area for future study.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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## SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.celrep.2019.07.036>.

## ACKNOWLEDGMENTS

This work was supported by the NIH grants GM127954 and CA222349, the Hyundai Hope On Wheels, the Four Diamonds, and the Lois High Berstler Research Endowment Fund. Authors would like to thank Nate Sheaffer, Joseph Bednarczyk, and Jade Vogel from the Penn State College of Medicine Flow Cytometry Core for assistance with the flow cytometry; Thomas Abraham and Wade Edris of the Penn State College of Medicine Imaging Core for assis-

tance with the confocal microscopy; and Haiyan Zheng of the Rutgers University Biological Mass Spectrometry Facility for assistance with the proteomic analysis.

## AUTHOR CONTRIBUTIONS

Z.T. and Y.T. performed the experiments and analyzed the data; H.H., T.H., C.C., H.C., X.L., and M.M.Y. assisted in collecting and analyzing the data and preparing the manuscript; Z.T., Y.T., M.M.Y., and H.-G.W. designed the experiments, discussed the results, and wrote the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: January 5, 2019

Revised: June 3, 2019

Accepted: July 12, 2019

Published: August 13, 2019

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## STAR★METHODS

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
rabbit polyclonal anti-Atg2A	MBL International	Cat# PD041; RRID: AB_2810871
rabbit polyclonal anti-Atg2B	Proteintech Group	Cat# 25155-1-AP; RRID: AB_2810874
rabbit monoclonal anti-Atg9A	Cell Signaling Technology	Cat# 13509; RRID: AB_2798241
mouse monoclonal anti- $\alpha$ -tubulin	Sigma-Aldrich	Cat# T5168; RRID: AB_477579
mouse monoclonal $\beta$ -actin	Sigma-Aldrich	Cat# A5441; RRID: AB_476744
rabbit polyclonal anti-GFP	Abcam	Cat# ab6556; RRID: AB_305564
mouse monoclonal anti-GST	Santa Cruz Biotechnology	Cat# sc-138; RRID: AB_627677
rabbit monoclonal anti-HSP90	Cell Signaling Technology	Cat# 4877; RRID: AB_2233307
rabbit monoclonal anti-LC3B	Cell Signaling Technology	Cat# 3868; RRID: AB_2137707
rabbit monoclonal anti-Atg13	Cell Signaling Technology	Cat# 13273; RRID: AB_2798169
rabbit polyclonal anti-LC3	Novus Biologicals	Cat# NB100-2220; RRID: AB_10003146
mouse monoclonal anti-mCherry	Abcam	Cat# ab125096; RRID: AB_11133266
mouse monoclonal anti-Myc-Tag	Cell Signaling Technology	Cat# 2276; RRID: AB_331783
normal mouse IgG	Santa Cruz Biotechnology	Cat# sc-2025; RRID: AB_737182
mouse monoclonal anti-TOM70	Santa Cruz Biotechnology	Cat# sc-390545; RRID: AB_2714192
guinea pig polyclonal anti-p62	American Research Product	Cat# 03-GP62-C; RRID: AB_1542690
mouse monoclonal anti-TOM20	Santa Cruz Biotechnology	Cat# sc-17764; RRID: AB_628381
mouse monoclonal anti-TOM40	Santa Cruz Biotechnology	Cat# sc-365467; RRID: AB_10847086
donkey anti-rabbit IgG IRDye 800CW	LiCOR	Cat# 925-32213; RRID: AB_2715510
donkey anti-rabbit IgG IRDye 680RD	LiCOR	Cat# 925-68073; RRID: AB_2716687
donkey anti-mouse IgG IRDye 800CW	LiCOR	Cat# 925-32212; RRID: AB_2716622
donkey anti-mouse IgG IRDye 680RD	LiCOR	Cat# 925-68072; RRID: AB_10953628
donkey anti-guinea pig IgG IRDye 680RD	LiCOR	Cat# 925-68077; RRID: AB_10956079
Nanogold-Fab goat anti-rabbit IgG	Nanoprobes	Cat# 2004; RRID: AB_2631182
goat anti-rabbit IgG Alexa Fluor 488	Life Technologies	Cat# A-11008; RRID: AB_143165
goat anti-rabbit IgG Alexa Fluor 568	Life Technologies	Cat# A-11031; RRID: AB_144696
goat anti-mouse IgG Alexa Fluor 647	Life Technologies	Cat# A-21238; RRID: AB_2535807
GFP-Trap	ChromoTek	Cat# gtma-20; RRID: AB_2631406
myc-Trap	ChromoTek	Cat# ytma-20; RRID: AB_2631370
Protein G Magnetic Beads	Bio-Rad	Cat# 1614021; RRID: AB_2021282
<b>Chemicals, Peptides, and Recombinant Proteins</b>		
Bafilomycin A1	LC Laboratories	Cat# B-1080
7-AAD	BioLegend	Cat# 420404
APC Annexin V	BioLegend	Cat# 640941
Rapamycin	LKT Laboratories	Cat# R0161
3-Methyladenine	Sigma-Aldrich	Cat# M9281
XF Plasma Membrane Permeabilizer	Seahorse Bioscience	Cat# 102504-100
HaloTag Alexa Fluor 488 Ligand	Promega	Cat# G1001
HaloTag TMR Ligand	VWR	Cat# PAG8251
16% Paraformaldehyde Aqueous Solution	Electron Microscopy Sciences	Cat# 15710
GoldEnhance EM (original)	Nanoprobes	Cat# 2113
Doxycycline hyclate	Sigma-Aldrich	Cat# D9891
L-Photo-Methionine	Thermo Fisher Scientific	Cat# 22615

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**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
L-Photo-Leucine	Thermo Fisher Scientific	Cat# 22610
Protease Inhibitor Cocktail	Sigma-Aldrich	Cat# P8340-5ML
Critical Commercial Assays		
BCA Protein Assay Kit	Thermo Fisher Scientific	Cat# 23252
Deposited Data		
Original proteomics dataset	Mendeley	<a href="https://data.mendeley.com/datasets/tn4p7g48hm/draft?a=b57fc569-4c2b-4012-9fee-f989309ebed2">https://data.mendeley.com/datasets/tn4p7g48hm/draft?a=b57fc569-4c2b-4012-9fee-f989309ebed2</a>
Experimental Models: Cell Lines		
U-2 OS	ATCC	HTB-96
HEK293T	ATCC	CRL-3216
HeLa	ATCC	CCL-2
Oligonucleotides		
Atg13 ON-TARGETplus SMARTpool - Human	Dharmacon	Cat# L-020765-01-0005
TOM40 ON-TARGETplus SMARTpool - Human	Dharmacon	Cat# L-012732-00-0005
sgAtg2A 5'-CGCTGCCCTTGTACAGATCG-3'	<a href="#">Tang et al., 2017</a>	N/A
sgAtg2B 5'-ATGGACTCCGAAAACGGCCA-3'	<a href="#">Tang et al., 2017</a>	N/A
sgAtg9A	Santa Cruz Biotechnology	Cat# sc-408011
sgTOM70 5'-GGCGCGTATACAGCGGGCTA-3' 5'-TAGCCCGCTGTATACGCGCC-3'	This paper	N/A
Recombinant DNA		
pCDH1-HTLC3	<a href="#">Takahashi et al., 2018</a>	N/A
pCDH1-EGFP-Atg2A(WT)	<a href="#">Tang et al., 2017</a>	N/A
pEGFP-Atg2A(WT)	This paper	N/A
pEGFP-MLD	This paper	N/A
pCDH1-dsRed-ER	This paper	N/A
pCDH1-EGFP-Atg2A( $\Delta$ MLD)	This paper	N/A
pEGFP-Atg2A( $\Delta$ MLD)	This paper	N/A
pEGFP-MLD(R-A)	This paper	N/A
pEGFP-MLD(L-A)	This paper	N/A
pCDH1-EGFP-Atg2A(L/R-A)	This paper	N/A
pEGFP-Atg2A(L/R-A)	This paper	N/A
pCDH1-EGFP-Atg2A(YFS-AAA)	This paper	N/A
pEGFP-Atg2A(YFS-AAA)	This paper	N/A
pCDH1-FRB-EGFP-Atg2A( $\Delta$ MLD)	This paper	N/A
pCDH1-TOM20-mRFP-FKBP	This paper	N/A
pCDH1-TOM70-mRFP-FKBP	This paper	N/A
pCAG-GST-WIP1	This paper	N/A
pCDH1-Atg9A-GFP	This paper	N/A
pBW-myc-Atg2A(WT)	This paper	N/A
pCDH1-EGFP-Atg2A( $\Delta$ 237-431)	This paper	N/A
pEGFP-Atg2A( $\Delta$ 237-431)	This paper	N/A
pBW-myc-Atg2A( $\Delta$ 237-431)	This paper	N/A
pCDH1-tet-on-EGFP-EF1-Puro-hUbc-rtTA	This paper	N/A
pCDH1-tet-on-EGFP-Atg2A(MLD)-EF1-Puro-hUbc-rtTA	This paper	N/A
pLenti-CRISPR-V2-sghAtg2A	<a href="#">Tang et al., 2017</a>	N/A
pLX-sghAtg2B	<a href="#">Tang et al., 2017</a>	N/A
LRG-sgTOM70	This paper	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and Algorithms		
Prism 7	GraphPad	<a href="https://www.graphpad.com">https://www.graphpad.com</a>
IMARIS	Oxford Instruments	<a href="https://imaris.oxinst.com">https://imaris.oxinst.com</a>
Velocity	PerkinElmer	<a href="https://www.perkinelmer.com/category/image-analysis-software">https://www.perkinelmer.com/category/image-analysis-software</a>
Huygens Professional	Scientific Volume Imaging	<a href="https://svi.nl/Huygens-Professional">https://svi.nl/Huygens-Professional</a>
Image Studio Lite Ver 5.2	Li-Cor	<a href="https://www.licor.com/bio/image-studio-lite/">https://www.licor.com/bio/image-studio-lite/</a>

**LEAD CONTACT AND MATERIALS AVAILABILITY**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Hong-Gang Wang ([hwang3@pennstatehealth.psu.edu](mailto:hwang3@pennstatehealth.psu.edu)). This study did not generate new unique reagents.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

U-2 OS (female), HeLa (female), and HEK293T (female) cells and indicated transfectants/transductants were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% FBS (VWR, 97068-091) and 1 × Antibiotic-Antimycotic solution (Corning, 30-004-CI). Cells were incubated at 37°C and 5% CO<sub>2</sub>.

**METHOD DETAILS****Transfection and transduction**

HEK293T cells were transfected using Calcium Phosphate method. Briefly, for 10 cm culture dish HEK293T cells, plasmid DNA (up to 15 μg) was diluted in 500 μL of 250 mM CaCl<sub>2</sub> solution. 500 μL of 2 × HEPES-buffered saline (140 mM NaCl, 1.5mM Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 50mM HEPES, pH 7.05) was added to DNA-CaCl<sub>2</sub> mixture dropwise while vortex and incubated for 10 minutes at room temperature before adding to cells. Plasmid DNA and siRNA transfection in U-2 OS and HEK293T cells were performed using Nucleofector Kit V (Lonza, VCA-1003), jetPRIME (Polyplus, 114-01) or FuGENE HD Transfection Reagent (Promega, E2311) per manufacturer's instructions. Lentivirus production and transduction were performed as described previously (Young et al., 2012).

**CRISPR/Cas9 genomic editing**

CRISPR/Cas9-mediated knockout was performed as described previously (Tang et al., 2017). Briefly, sgRNAs targeting human TOM70, Atg2A, and Atg2B were sub-cloned into Lenti-sgRNA-EFS-GFP (TOM70), pLenti-CRISPR-V2 (Atg2A), and pLX-sgRNA (Atg2B) vectors, respectively. CrAtg2A/B U-2 OS, HEK293T, and HeLa cells were generated by infecting cells with pLenti-CRISPR-V2-sgAtg2A lentivirus followed by 7 days of puromycin selection. Cells were then subjected to transduction with pLX-sgAtg2B lentivirus and blasticidin selection for 10 days. CrTOM70 HEK293T cells were generated by infecting cells with lentiCas9-Blast virus followed by 10 days of blasticidin selection. Cells were then subjected to transduction with Lenti-sgTOM70-EFS-GFP for 2 days and followed by GFP-positive FACS sorting. To generate Atg9A knockout cells, U-2 OS cells were transiently transfected with sgAtg9A-Cas9-P2A-GFP plasmid (Santa Cruz sc-408011) and followed by GFP-positive FACS sorting. Single clones were isolated, expanded, and screened for complete knockout by immunoblotting.

**Autophagic flux assay**

Cells were treated as described in the text prior to immunoblotting. The intensity of p62 and loading control was quantified using LI-COR Biosciences Image Studio software, and p62 levels were normalized to loading control. Basal autophagic flux (1) and starvation-induced autophagic flux (2) were calculated using the following formulas (Tooze et al., 2015): (1) [(complete media with BafA1 – complete media)/(complete media with BafA1)] × 100; (2) [(starvation media with BafA1 – starvation media)/(starvation media with BafA1)] × 100. All data were normalized to WT.

**FACS cell death analysis**

Cells were treated as described in the text. Cells were harvested by trypsinization and washed once in PBS prior to 5% Annexin-V and 7-AAD staining for 15 mins on ice. Cells were then washed once in Annexin-V binding buffer prior to FACS analysis.

**HaloTag-LC3 autophagosome completion assay**

The assay was performed as previously described (Takahashi et al., 2018). Briefly, cells were incubated at 37°C with AlexaFluor488-MIL in permeabilization buffer (3 nM XF-PMP in 1 × MAS buffer (220 mM mannitol, 70 mM sucrose, 10 mM KH<sub>2</sub>PO<sub>4</sub>, 5 mM MgCl<sub>2</sub>,

2 mM HEPES, 1 mM EGTA)) for 15 minutes. Equal volume of 2 × fixative (8% PFA and 400 mM sucrose in 1 × PBS) was added to cells and incubated at room temperature for 7 minutes. Cells were then washed with PBS and incubated with tetramethylrhodamine (TMR)-MPL in PBS at room temperature for 30 minutes. Finally, cells were washed with PSB before confocal microscopy.

### Immunoblotting

Cells were collected on ice by scraping and washed with ice-cold PBS and then resuspended and incubated in RIPA buffer (25 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail) for 30 minutes on ice. Cell lysate supernatant was collected after 15 minutes 20,000 × g centrifugation. Protein concentrations were determined using BCA assay. Equal amount protein from each sample was denatured using 2 × Laemmli buffer (4% SDS, 20% glycerol, 120 mM Tris-HCl pH 6.8, 0.02% bromophenol blue, 10% 2-mercaptoethanol). Samples were then heated (100°C, except 70°C for Atg9A) for 10 minutes before electrophoresis. Proteins were then transferred to PVDF membrane overnight followed by blocking and antibody incubation. Membranes were imaged using Li-Cor Odyssey CLx Infrared Imaging System.

### Co-immunoprecipitation

Co-IP using transfected HEK293T cells was performed as described previously with minor optimization (Tang and Takahashi, 2018). For TOM40 co-IP, cells were lysed in NP-40 lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40). For Atg9A-GFP co-IP, cells were lysed in TX-100 lysis buffer (50 mM Tris-HCl pH 7.4, 75 mM NaCl, 0.8% TX-100). For Atg9A co-IP, cells were homogenized in PBS by going through 27.5-gauge needle 20 times. Triton X-100 was then added to cell homogenate (0.01% final concentration). For WIPI4 co-IP, lysis buffer recipe was obtained from previous publication (Zheng et al., 2017). Cells were lysed for 30 minutes at 4°C while rotating. Cell lysates were then centrifuged for 15 minutes (20,000 × g, except 4000 × g for Atg9A) and supernatants were collected and subjected to BCA assay. 500 μg protein lysate from each sample was incubated with 25 μL post-washing GFP-Trap\_MA beads for 3 hours at 4°C while rotating. Beads were then washed 3 times with washing buffer (lysis buffer with 10% original detergent concentration) before immunoblotting analysis. For TOM40-Atg2A endogenous co-IP, magnetic protein-G beads (Bio-Rad, 1614021) were incubated with 10 μg TOM40 antibody (Santa Cruz) or normal mouse IgG in diluted (50% original detergent concentration) NP-40 lysis buffer for 2 hours at room temperature. Antibody-protein-G bead conjugates were washed 3 times in washing buffer prior to incubation with cell lysates (NP-40 lysis buffer) for 2 hours at room temperature. Beads were then washed 3 times with washing buffer (lysis buffer with 10% original detergent concentration) before immunoblotting analysis.

### Fluorescence microscopy

Cells were seeded on 8-well chamber slides (Fisher Scientific, 12-565-1) overnight and subjected to indicated treatments. For TOM20 and Atg9A immunostaining, cells were fixed in 4% paraformaldehyde-PBS for 20 minutes in dark at room temperature and then subjected to 0.15% Triton X-100-PBS for 20 minutes or 100 μg/ml digitonin-PBS for 10 minutes at room temperature. Cells were rinsed with PBS 3 times and then incubated with 10% normal goat serum-PBS for 1 h at room temperature before overnight incubation of indicated primary antibodies in 1% normal goat serum-PBS at 4°C. Cells were washed 3 times with PBS and then incubated with indicated secondary antibodies for 1 hour in dark at room temperature. Cells were rinsed 3 times with PBS before confocal microscopy. For endogenous LC3B immunostaining, cells were fixed in 4% paraformaldehyde-PBS briefly at room temperature (approximately 15–30 s) and then incubated with ice-cold methanol for 10 mins at –20°C. Cells were rinsed with PBS 3 times before 1.5 hours incubation with anti-LC3B antibody in 1% BSA-0.1% TWEEN-20-PBS. Cells were then rinsed with 0.1% TWEEN-20-PBS 3 times and subjected to incubation with indicated secondary antibodies and confocal microscopy. For HaloTag-LC3 MPL staining, cells were incubated with MPL in PBS for 1 hour and then washed 3 times with PBS. Leica AOBSP8 with 63 × water-immersion lens or 63 × oil-immersion lens was used to analyze samples. Fluorescent images were then deconvolved, processed, and quantified using Huygens (Scientific Volume Imaging), IMARIS (Oxford Instruments), and Volocity (PerkinElmer) software, respectively.

### Transmission electron microscopy, immuno-gold electron microscopy, and correlative light electron microscopy

TEM, immuno-TEM, and CLEM were performed as described previously (Takahashi et al., 2018). Briefly, for TEM, cells were seeded on plastic coverslip (Thermo Scientific 174950) in 24-well plate overnight. Cells were then treated with indicated conditions followed by 1 hour fixation in dark at room temperature (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3). Samples were then incubated with osmium fixation buffer (1% osmium tetroxide with or without 1.5% potassium ferrocyanide in 0.1 M sodium cacodylate, pH 7.3) for 1 hour followed by serial ethanol dehydration and resin embedding (Electron Microscopy Sciences, 14120). 70 nm sample sections were mounted on mesh copper grids followed by uranyl acetate and lead citrate staining before analyzed using JEOL JEM 1400 transmission electron microscope. For immuno-TEM, cells were seeded on plastic coverslip in 24-well plate overnight and treated with indicated conditions followed by 2 hours fixation (4% paraformaldehyde in phosphate buffer (PB), pH 7.4) in dark at room temperature. Samples were incubated in permeabilization buffer (0.25% saponin in PB) for 30 minutes followed by 1 hour incubation with blocking buffer (10% BSA, 10% normal goat serum, 0.1% cold water fish gelatin, 0.1% saponin in PB). Samples were incubated with the primary antibody at 4°C overnight and incubated with the gold-conjugated secondary antibody at room temperature for 1 hour followed by 10 minutes fixation (1% glutaraldehyde in PB), 15 minutes washing (50 mM glycine in PBS), and signal intensification using GoldEnhance EM for 5 minutes. Samples were then processed as described above. For CLEM, cells were seeded on Gridded Glass Bottom Dish (MatTek, P35G-1/5-14-C-GRID) overnight. Cells were then treated with

indicated conditions and subjected to HaloTag-LC3 autophagosome completion assay or solely MPL(TMR) staining. Samples were analyzed by confocal microscopy and cells of interest were then processed for TEM, identified by grid number.

#### **Photo-amino acid, UV crosslinking, and proteomic analysis**

HEK293T cells were transduced with pCDH1-tet-on-EGFP or pCDH1-tet-on-EGFP-MLD lentivirus. Cells were then selected with 2  $\mu\text{g}/\text{ml}$  puromycin for 4 days.  $9 \times 10^6$  cells were seeded on 10 cm dish overnight and medium was replaced with 10 mL photo-leucine and photo-methionine containing medium in the presence of 2  $\mu\text{g}/\text{ml}$  doxycycline hyclate for 24 hours. Cells were then placed 2 cm under a 20 W 365 nm UV bench lamp (Blak-Ray XX-20BLB) and irradiated for 20 minutes. Cells were harvested and lysed on ice using NP-40 co-IP lysis buffer. Protein concentrations were determined using BCA assay. 500  $\mu\text{g}$  lysate from each sample was incubated with 25  $\mu\text{L}$  of post-washed GFP-Trap\_MA beads for 3 hours at 4°C while rotating. Beads were then washed for 10 times and proteins were eluted with 25  $\mu\text{L}$  2  $\times$  laemmli buffer and 100°C heating for 10 minutes. Samples were then submitted for proteomic analysis at the Rutgers University Biological Mass Spectrometry Facility.

#### **Rapamycin-induced oligomer formation system of FRB-FKBP fusion proteins**

U-2 OS cells were transduced with lentivirus encoding FRB-EGFP-Atg2A( $\Delta$ MLD) followed by TOM20-mRFP-FKBP or TOM70-mRFP-FKBP prior to EGFP and mRFP double positive FACS sorting. Cells were treated with DMSO or 150 nM rapamycin for 8 hours prior to immunostaining and confocal microscopy.

#### **QUANTIFICATION AND STATISTICAL ANALYSIS**

Fluorescence microscopy quantifications were performed using Velocity and IMARIS software. Immunoblotting quantifications were performed using Image Studio software.

Statistical significance was determined using Graph Pad Prism 7.0. Statistical computation, threshold for significance, and  $n$  numbers were indicated on each figure.

#### **DATA AND CODE AVAILABILITY**

Original proteomics data for [Figure 5A](#) and [Table S1](#) in the paper is available at Mendeley Data <https://doi.org/10.17632/tn4p7g48hm.2>.

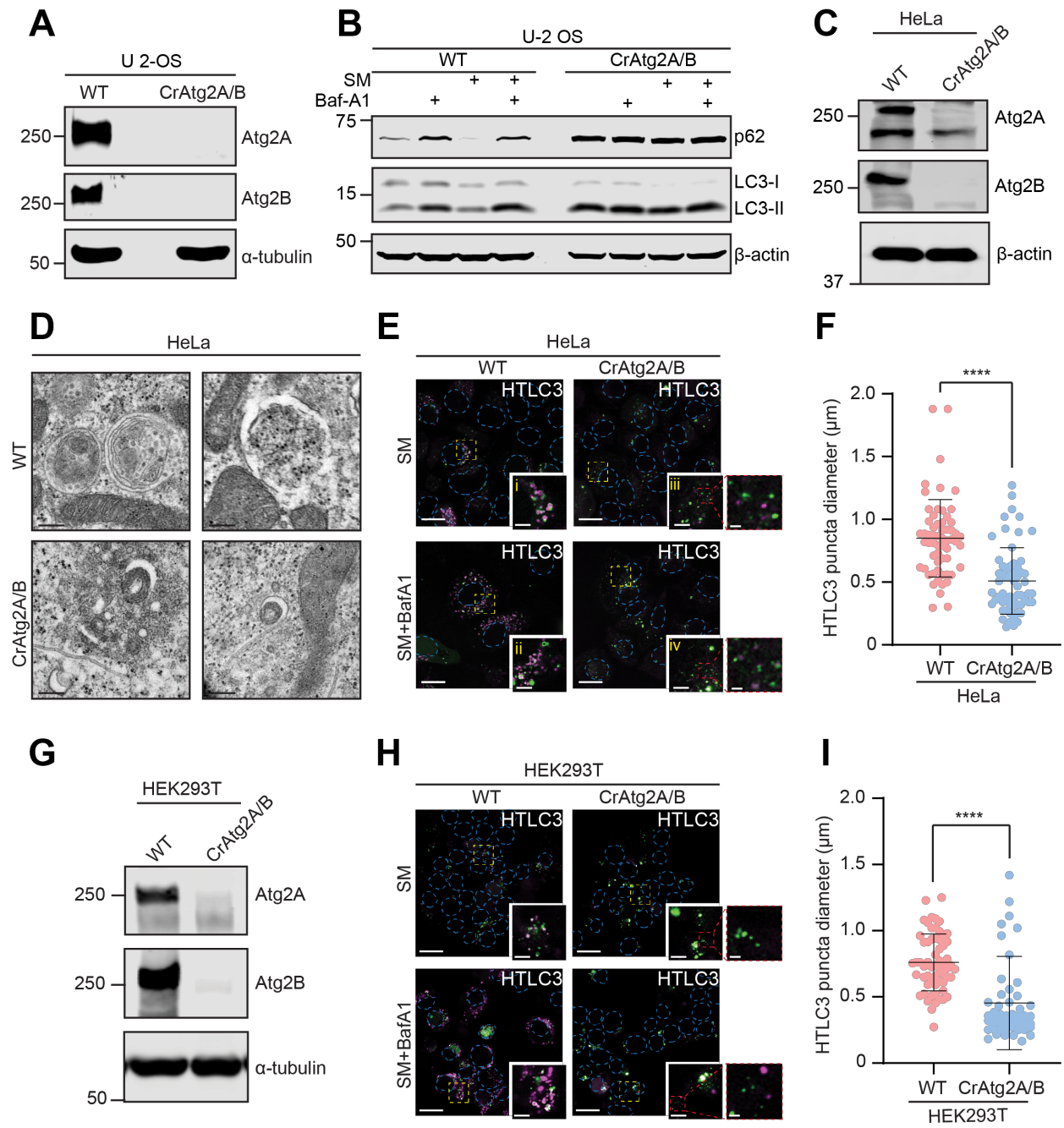
**Cell Reports, Volume 28**

**Supplemental Information**

**TOM40 Targets Atg2 to Mitochondria-Associated**

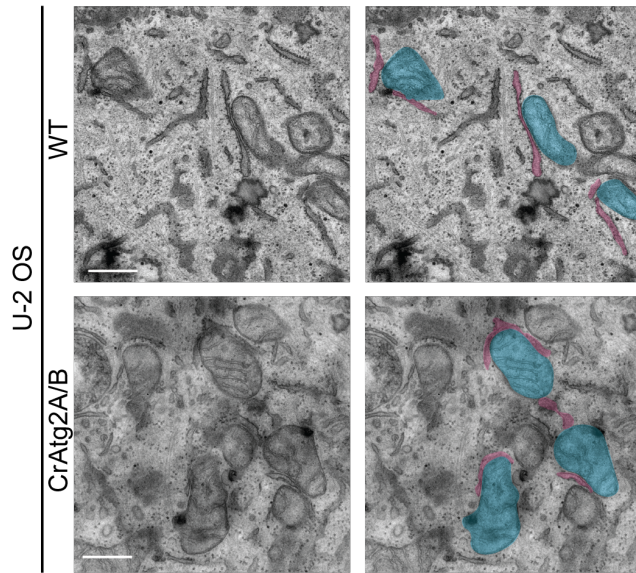
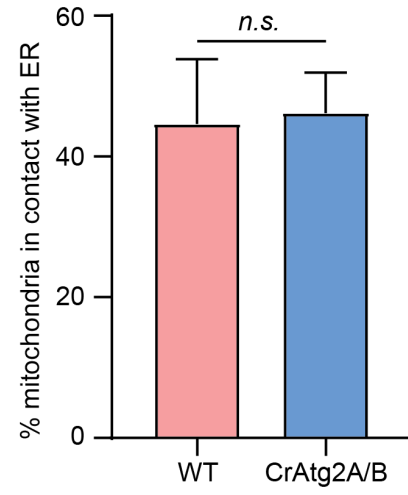
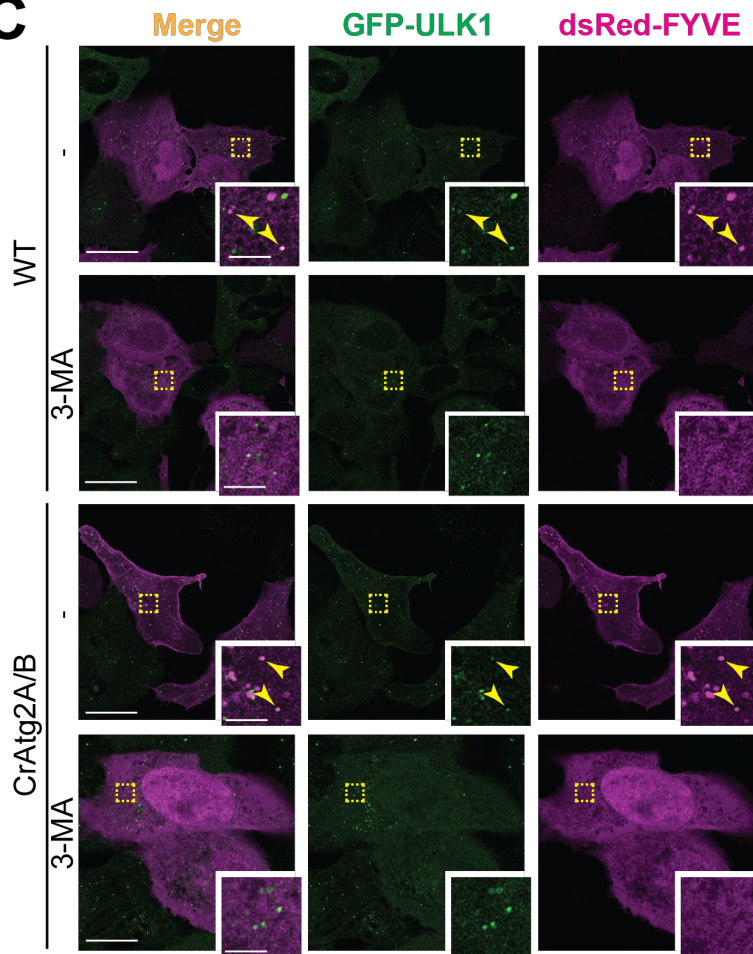
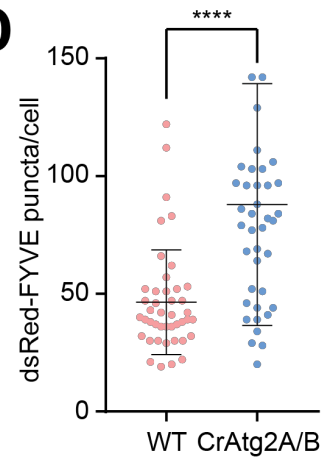
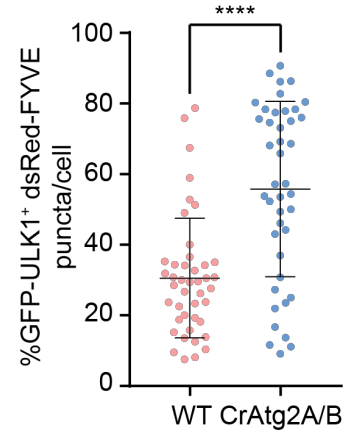
**ER Membranes for Phagophore Expansion**

**Zhenyuan Tang, Yoshinori Takahashi, Haiyan He, Tatsuya Hattori, Chong Chen, Xinwen Liang, Han Chen, Megan M. Young, and Hong-Gang Wang**



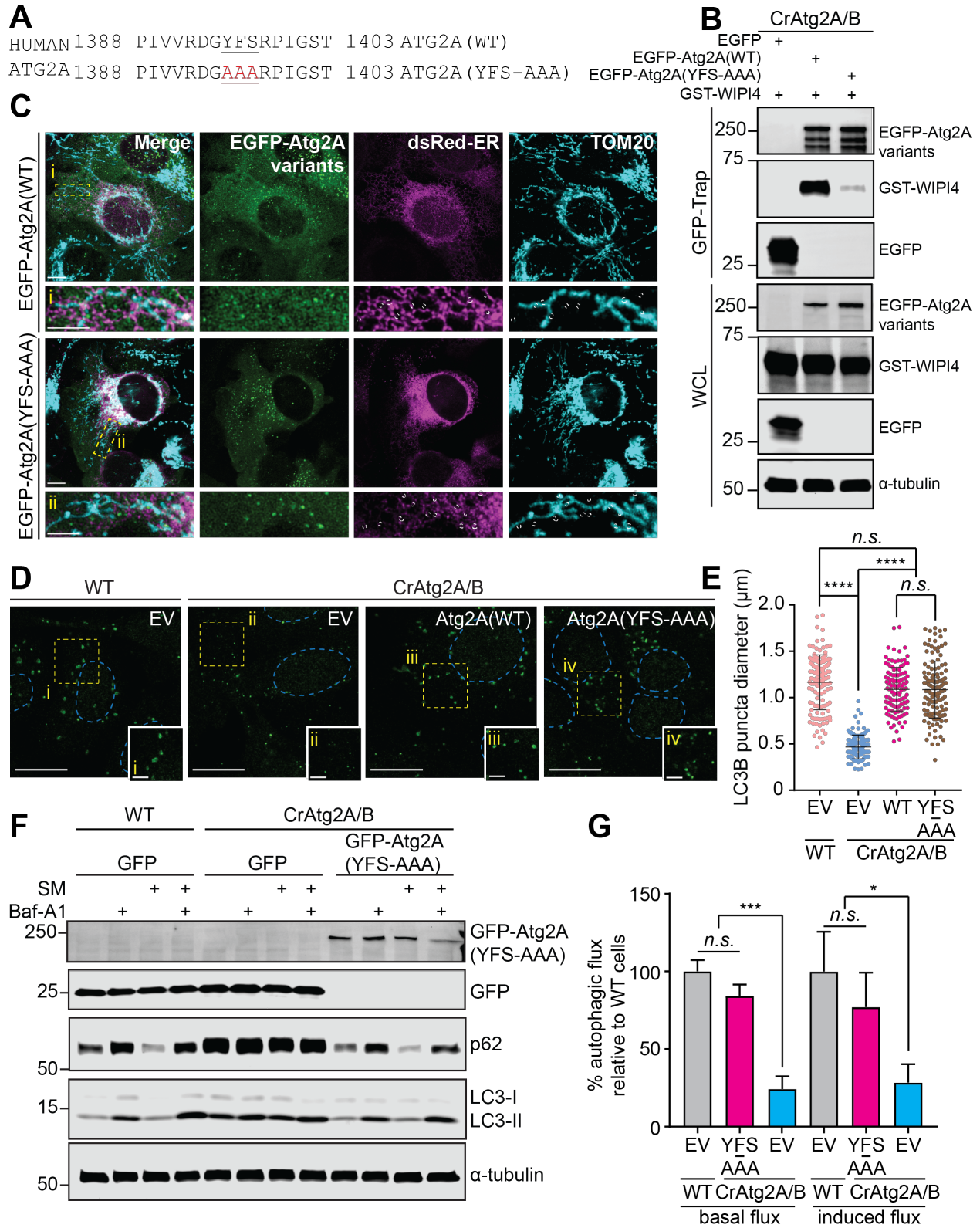
**Figure S1**

**Figure S1. Loss of Atg2A/B impairs autophagic flux and results in small autophagosomes. Related to Figure 1. (A, C, G)** U-2 OS **(A)**, HeLa **(C)**, and HEK293T **(G)** cells were transduced with lentiviruses encoding Cas9 and sgRNAs targeting Atg2A and Atg2B (CrAtg2A/B) and subjected to immunoblotting with the indicated antibodies. **(B)** WT and CrAtg2A/B U-2 OS cells were incubated in complete medium (CM) or starvation medium (SM) for 2 hours in the presence or absence of 100 nM Baf-A1 and subjected to immunoblotting with the indicated antibodies. **(D)** WT and CrAtg2A/B HeLa cells were starved for 2 hours and subjected to transmission electron microscopy. Representative autophagic structures are shown. Red arrowheads indicate autophagic structures. Scale bars represent 200 nm. **(E, H)** HT-LC3-expressing WT and CrAtg2A/B HeLa **(E)** and HEK293T **(H)** cells were incubated in CM or SM in the presence or absence of 100 nM Baf-A1 for 2 hours and subjected to the HT-LC3 autophagosome completion assay followed by confocal microscopy. Magnified images of the boxed areas are shown in the insets (yellow boxes) and the lower bottom right panels (red boxes). Scale bars represent 20  $\mu\text{m}$ , 5  $\mu\text{m}$  (yellow box) and 1  $\mu\text{m}$  (red box) in the magnified images. **(F, I)** The diameters of HT-LC3 foci randomly selected from the starved WT and CrAtg2A/B HeLa **(F)** and HEK293T **(I)** cells were quantified using the Velocity software (n=60). Statistical significance was determined by t-test. All values are mean  $\pm$  SD. \*\*\*\* $p \leq 0.0001$ .

**A****B****C****D****E****Figure S2**



**Figure S2. Atg2A/B is not essential for mitochondria-ER contact site establishment and PI3P distribution. Related to Figure 2. (A)** WT and CrAtg2A/B U-2 OS cells were starved for 2 hours and subjected for transmission electron microscopy. Blue and magenta labeled areas represent mitochondria and ER, respectively. Scale bars represent 500 nm. **(B)** The percentage of mitochondria that contact with ER in WT and CrAtg2A/B U-2 OS cells were quantified. (n=1191 mitochondria from 19 WT cells; n=1633 mitochondria from 19 CrAtg2A/B cells). **(C)** GFP-ULK1 stable expressing WT and CrAtg2A/B U-2 OS cells were transiently transfected with dsRed-FYVE for 24 hours, starved in the presence or absence of 10 mM 3-MA for 4 hours, and analyzed by confocal microscopy. Magnified images of the boxed areas are shown in the insets. Scale bars represent 30  $\mu\text{m}$ , and 5  $\mu\text{m}$  in the magnified images. **(D, E)** Total dsRed-FYVE puncta **(D)** and the percentage of GFP-ULK1-positive dsRed-FYVE puncta **(E)** per cell in WT and CrAtg2A/B U-2 OS cells were quantified (n=42). Statistical significance was determined by t-test. All values are mean  $\pm$  SD. *n.s.* not significant. \*\*\*\* $p \leq 0.0001$ .



**Figure S3. The interaction of Atg2A with WIPI4 is dispensable for both autophagosome formation and autophagic flux. Related to Figure 2. (A)** The amino acid sequence of the WIPI4 interacting region of Atg2A is shown. The mutated region and substituted amino acids are indicated by underlines and red characters. **(B)** CrAtg2A/B HEK293T cells were transfected with the indicated plasmids for 24 hours and subjected to immunoprecipitation with GFP-Trap beads followed by immunoblotting with the indicated antibodies. **(C)** CrAtg2A/B U-2 OS cells stably expressing dsRed-ER and the indicated EGFP-Atg2A variant were starved for 2 hours, stained for TOM20 and analyzed by confocal microscopy. The magnified images in the boxed areas are shown in the lower panels. Scale bars represent 10  $\mu\text{m}$ , and 5  $\mu\text{m}$  in the magnified images. **(D)** CrAtg2A/B cells U2-OS cells stably expressing the indicated EGFP-Atg2A variants were starved for 2 hours, stained for LC3 and analyzed by confocal microscopy. Magnified images in the boxed areas are shown in the insets. Scale bars represent 20  $\mu\text{m}$ , and 5  $\mu\text{m}$  in the magnified images. EV, WT, YFS represent EGFP-empty vector, EGFP-Atg2A(WT), EGFP-Atg2A(YFS), respectively. **(E)** The diameters of LC3 foci were randomly selected and quantified using the Velocity software (n=130). EV, WT, YFS represent EGFP-empty vector, EGFP-Atg2A(WT), EGFP-Atg2A(YFS), respectively. **(F, G)** The indicated U-2 OS cells were incubated in CM or SM for 2 hours in the presence or absence of 100 nM Baf-A1 and subjected to immunoblotting with the indicated antibodies **(F)**. Basal and starvation-induced autophagic flux in the indicated U-2 OS stable transductants were quantified as described in the Methods (n=3) **(F)**. Statistical significance was determined by one-way ANOVA followed by Dunn's multiple comparisons test. All values are mean  $\pm$  SD. *n.s.* not significant. \* $p \leq 0.05$ ; \*\*\* $p \leq 0.001$ ; \*\*\*\* $p \leq 0.0001$ .

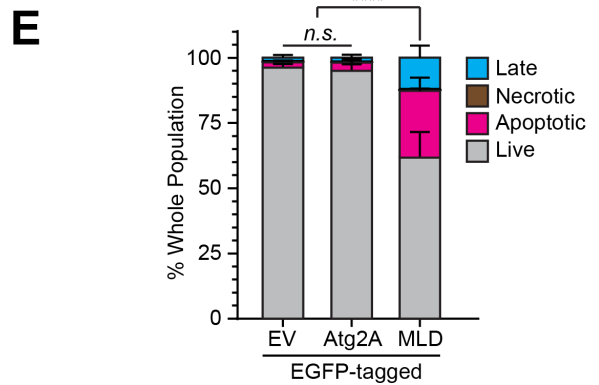
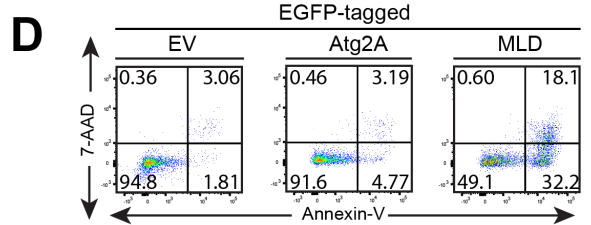
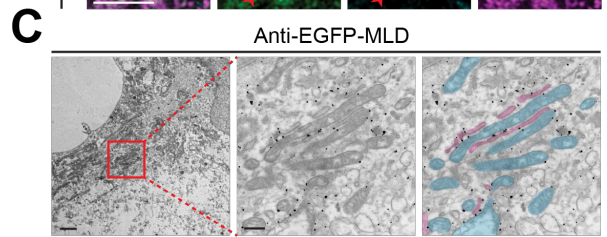
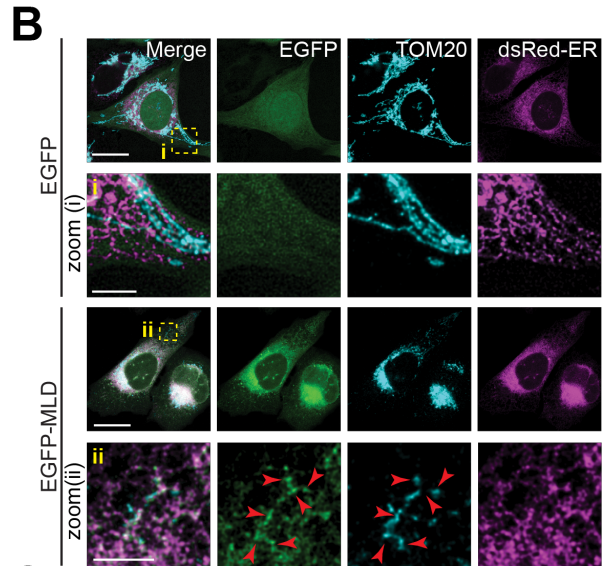
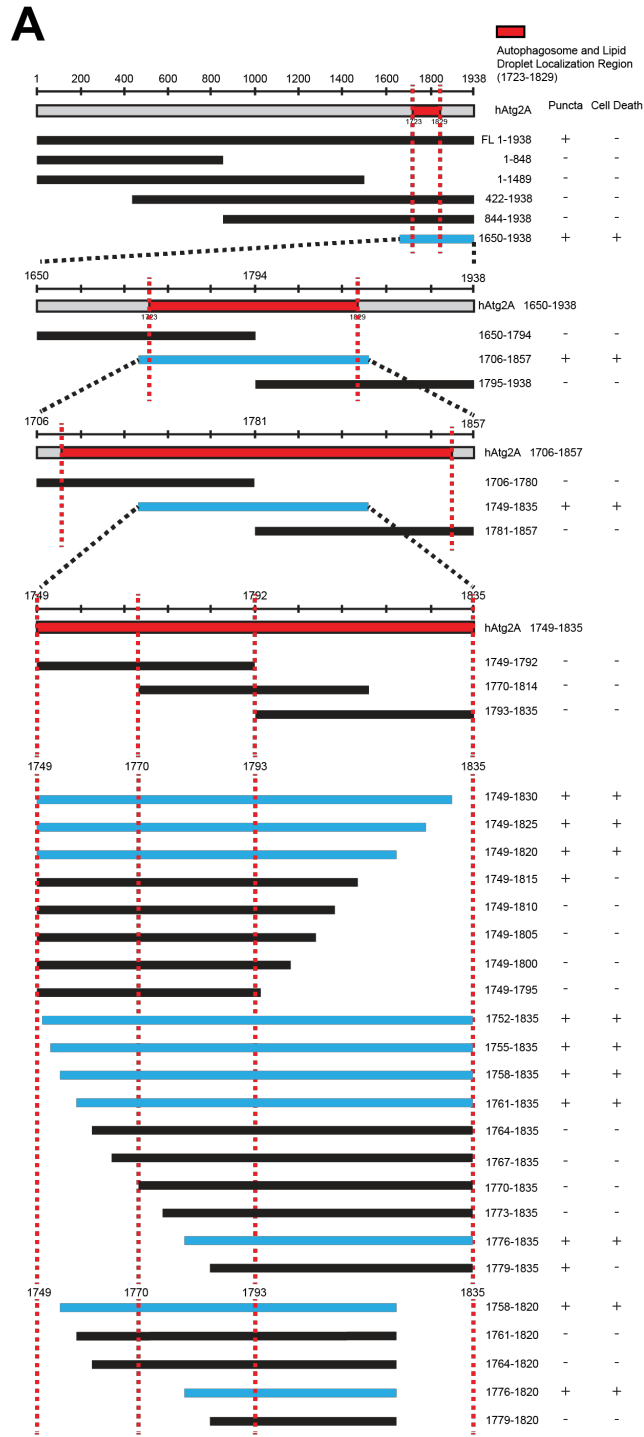
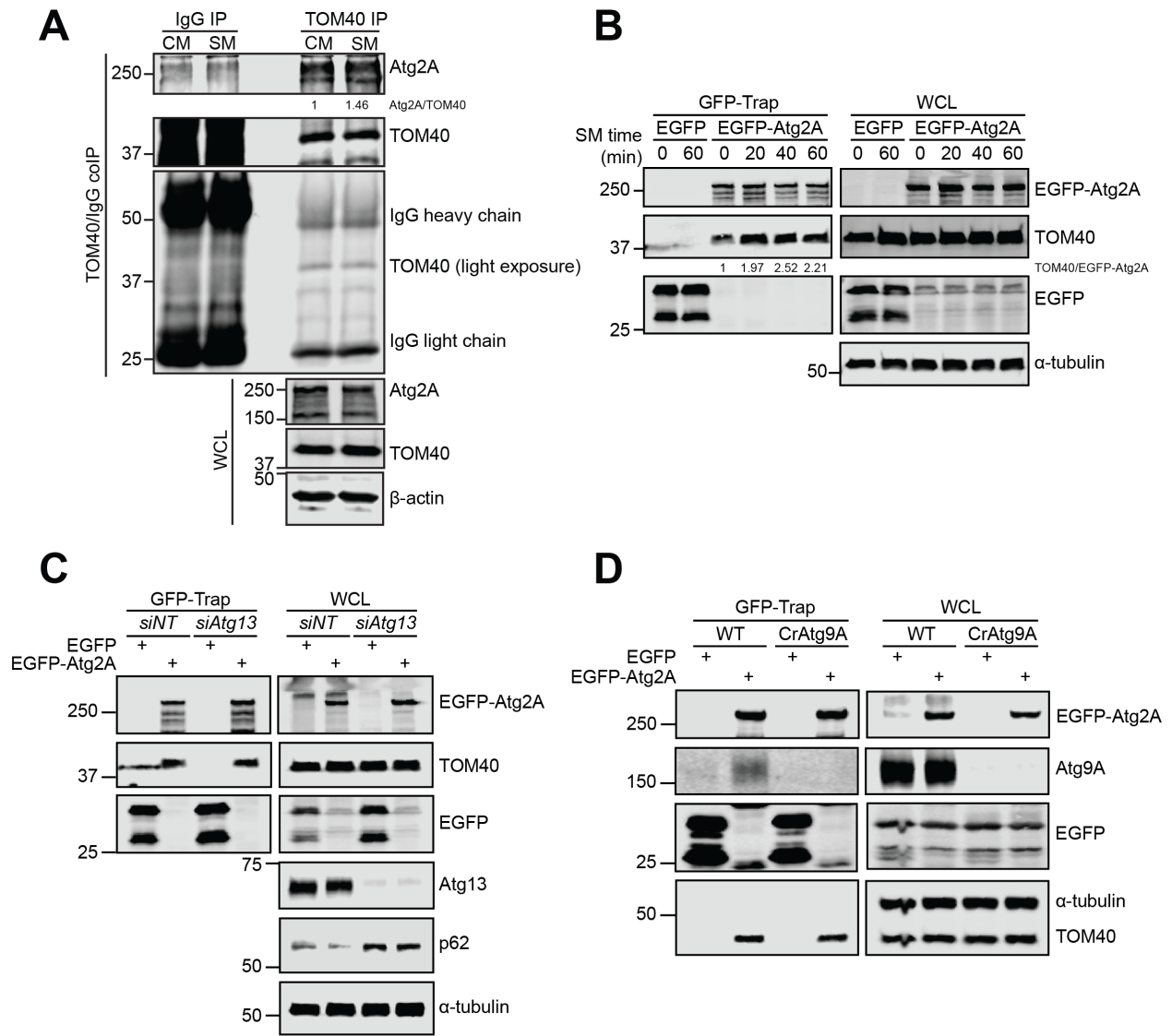


Figure S4

**Figure S4. Atg2A(1776-1820) is sufficient for MAM localization. Related to Figure 3. (A)** EGFP-tagged human Atg2A was truncated from N- and/or C-terminus sequentially. CrAtg2A/B HEK293T cells were transiently transfected with Atg2A mutants for 24 hours. Cytosolic EGFP-Atg2A mutant punctuation and cell death were analyzed by fluorescence microscopy. The red box represents the previously identified autophagosome and lipid localization region on human Atg2A (AK Velikkakath et al, MBoC, 2012). **(B)** Schematic illustration of MAM localization domain (MLD) of Atg2A. **(C)** CrAtg2A/B U-2 OS cells stably expressing dsRed-ER were transiently transfected with EGFP or EGFP-MLD and subjected to immunofluorescence confocal microscopy using anti-TOM20 antibody. Magnified images in the boxed areas are shown in the lower panels. Scale bars represent 20  $\mu\text{m}$ , and 5  $\mu\text{m}$  in the magnified images. **(D, E)** CrAtg2A/B U-2 OS cells were transiently transfected with EGFP or EGFP-MLD and subjected to immunoelectron microscopy using anti-GFP antibody **(D)** or Annexin-V and 7-AAD staining and flow cytometry analysis **(E)**. The magnified image (red box) is shown on the right panel. Blue and magenta labeled areas represent mitochondria and ER, respectively. Scale bars represent 2000 nm, 500 nm in the magnified image **(D)**. Statistical significance was determined by one-way ANOVA followed by Dunn's multiple comparisons test. All values are mean  $\pm$  SD. *n.s.* not significant. \*\*\*\* $p \leq 0.0001$ .



**Figure S5**

**Figure S5. Atg2A-TOM40 interaction is enhanced by starvation but does not require upstream ATG machinery. Related to Figure 3. (A)** WT HEK293T cells were incubated in CM or SM for 1 hour and subjected to immunoprecipitation with control IgG or TOM40 antibody followed by immunoblotting with the indicated antibodies. **(B)** CrAtg2A/B HEK293T cells were transfected with EGFP or EGFP-Atg2A for 24 hours and incubated in SM for the indicated time prior to immunoprecipitation with GFP-Trap and followed by immunoblotting with the indicated antibodies. **(C)** CrAtg2A/B HEK293T cells were transfected with non-targeting (siNT) or Atg13 (siAtg13) siRNA for 48 hours followed by EGFP or EGFP-Atg2A transfection for 24 hours prior to immunoprecipitation with GFP-Trap and followed by immunoblotting with the indicated antibodies. **(D)** WT and CrAtg9A HEK293T cells were transfected with EGPF or EGFP-Atg2A for 24 hours and subjected to immunoprecipitation with GFP-Trap and followed by immunoblotting with the indicated antibodies.

**A**

<sup>I M F L Q T A</sup>  
 GRLIRGLQRGAASFGSS<sup>TTR</sup>TASAALELSNRLVQAIQATAETVYDILS

H. sapiens 1776 GRLMRGLQRGAASFGSS<sup>TTR</sup>TASAALELSNRLVQAIQATAETVYDILS 1820

R. norvegicus 1754 GRLIRGLQRGAASFGSS<sup>TTR</sup>TASAALELSNRLVQAIQATAETVYDILS 1798 Query\_9407

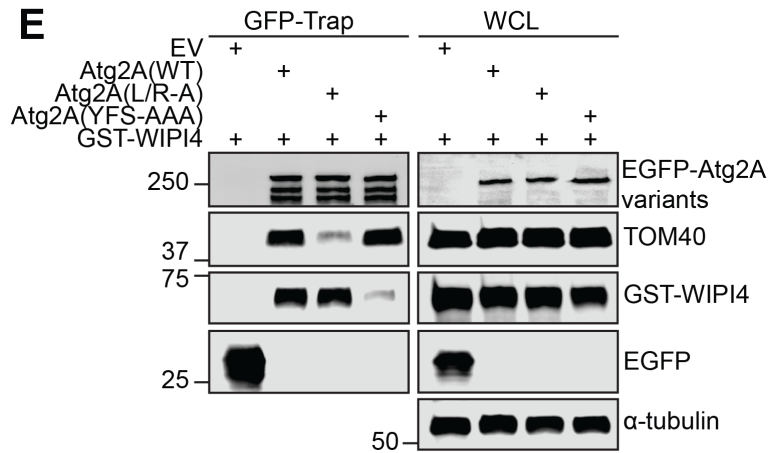
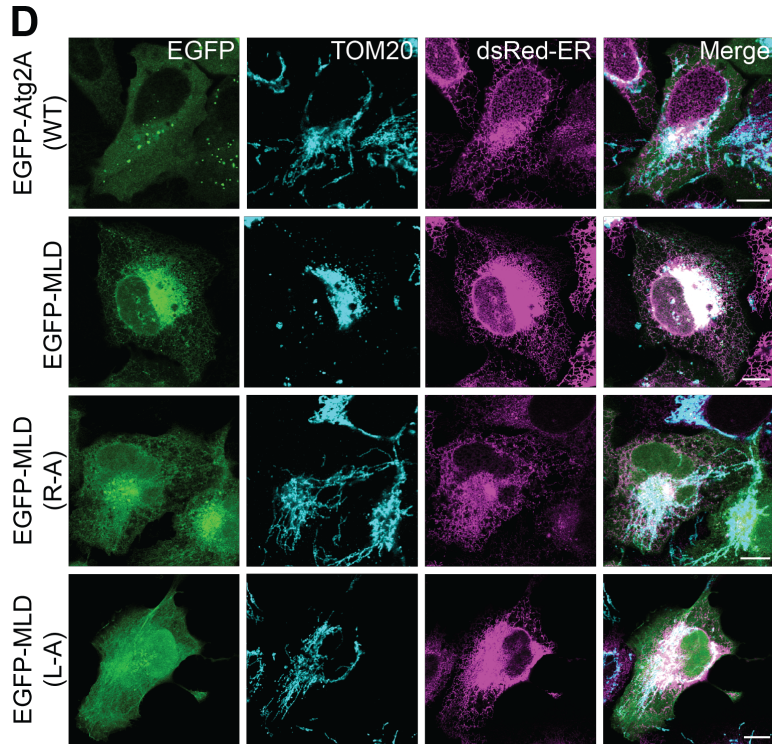
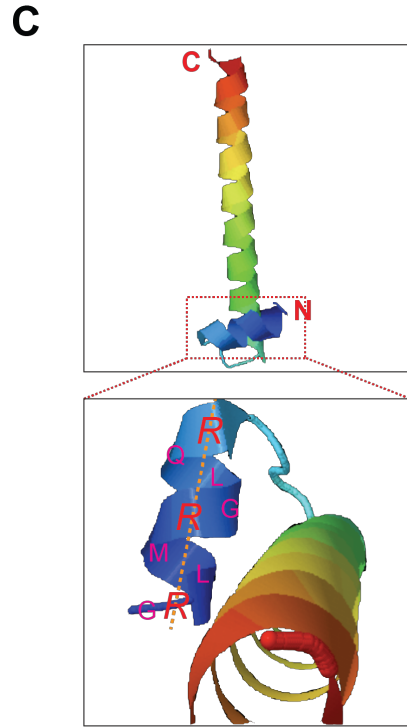
M. musculus 1752 GRLIRGLQRGAASFGSS<sup>TTR</sup>TASAALELSNRLVQAIQATAETVYDILS 1796 Query\_9407

D. rerio 1863 GR<sup>IIR</sup>GLQRGAASFGTSTASAALELSNRLVQAIQATAETVYDILS 1907 Query\_9407

D. melanogaster 1748 GR<sup>IIR</sup>GFQLGAQSFTARTALAALELTSR<sup>I</sup>IHLLQFTAETTFDMLS 1792 Query\_9407

**B**

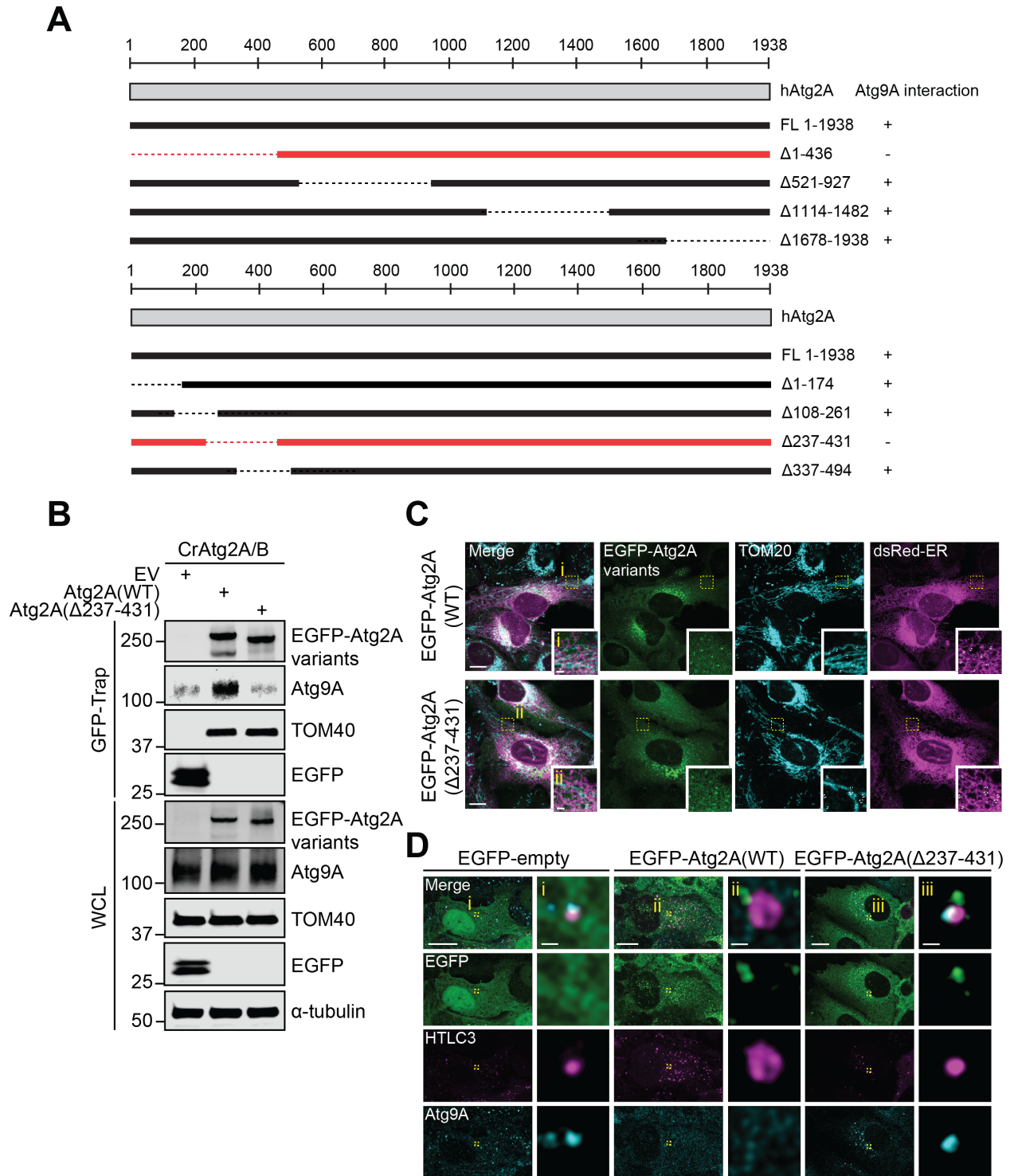
Secondary Sequence: GRLMRGLQRGAASFGSS<sup>\*</sup>TASAALELSNRLVQAIQATAETVYDILS Helix  
 1 5 10 15 20 25 30 35 40 45 Coil



**Figure S6**



**Figure S6. The arginine and leucine residues are crucial for MLD localization. Related to Figure 3. (A)** Protein sequence alignment of Atg2A MLD among higher eukaryotes. **(B, C)** Protein secondary **(B)** and tertiary **(C)** structures of MLD were predicted using QUARK ab initio protein structure prediction. Asterisks in **B** indicate the identified crucial amino acid residues for MLD localization. **(D)** dsRed-ER-expressing CrAtg2A/B U2-OS cells were transfected with the indicated EGFP-tagged wild-type or mutant Atg2A for 24 hours, stained for TOM20 and analyzed by confocal microscopy. Scale bars represent 10  $\mu$ m. **(E)** CrAtg2A/B HEK293T cells were transfected with the indicated plasmids for 24 hours and subjected to immunoprecipitation with GFP-Trap beads followed by immunoblotting with the indicated antibodies.



**Figure S7**

**Figure S7. Atg2A is dispensable for the anterograde trafficking of Atg9A. Related to Figure 7. (A)** EGFP-tagged human Atg2A was truncated as indicated. Co-immunoprecipitation analyses were performed using GFP-Trap beads to determine the interaction of the indicated Atg2A mutants with Atg9A. **(B)** CrAtg2A/B HEK293T cells were transfected with EGFP-Atg2A variants for 24 hours and subjected to immunoprecipitation with GFP-Trap beads followed by immunoblotting with the indicated antibodies. **(C, D)** CrAtg2A/B U-2 OS cells stably expressing the indicated plasmids were starved for 2 hours, stained for TOM20 **(C)** or Atg9A and MPL (TMR) **(D)** and analyzed by confocal microscopy. Magnified images in the boxed areas are shown in the insets **(C)** and right panels **(D)**. Scale bars represent 20  $\mu\text{m}$ , 2.5  $\mu\text{m}$  in the insets in **C**, and 0.5  $\mu\text{m}$  in the magnified images in **D**.

**Table S1. List of EGFP-MLD interacting partner candidates. Related to Figure 3.** Protein hits are normalized to their corresponding EGFP- or EGFP-MLD peptide number. All candidates, with over 5-fold enrichment, are listed.

gene name	EGFP	EGFP-MLD	EGFP	EGFP-MLD	relative fold-change	Subcellular Localization
	# of peptides		normalize to bait			
EGFP	604	1485				
PHB	11	186	0.01821192	0.12525253	6.877502296	Mit inner
EMC1	5	142	0.00827815	0.0956229	11.55124579	ER
IMMT	3	104	0.00496689	0.07003367	14.10011223	Mit inner
ATAD3B	5	66	0.00827815	0.04444444	5.368888889	Mit inner
TOMM40	2	59	0.00331126	0.03973064	11.9986532	Mit outer
GOLGA5		44	0	0.02962963		Golgi
ALDH3A2	3	41	0.00496689	0.02760943	5.558698092	ER
FAF2	3	38	0.00496689	0.02558923	5.151964085	ER
SAMM50		38	0	0.02558923		Mit outer
RAB3GAP1		34	0	0.02289562		Others
TMEM43	2	31	0.00331126	0.02087542	6.304377104	ER
CHCHD3	1	29	0.00165563	0.01952862	11.7952862	Mit inner
ANKLE2	1	28	0.00165563	0.01885522	11.38855219	ER
MMGT1	1	27	0.00165563	0.01818182	10.98181818	ER
AKAP1		27	0	0.01818182		Mit outer
ZMPSTE24	1	25	0.00165563	0.01683502	10.16835017	ER
TOR1AIP2	1	25	0.00165563	0.01683502	10.16835017	ER
LEMD3		26	0	0.01750842		Nucleus
DNAJC11		26	0	0.01750842		Mit outer
MTX2		24	0	0.01616162		Mit outer
EMC3	1	23	0.00165563	0.01548822	9.354882155	ER
CLCC1	1	23	0.00165563	0.01548822	9.354882155	ER
DNM1L	1	22	0.00165563	0.01481481	8.948148148	Mit outer
ARFGAP1	1	19	0.00165563	0.01279461	7.727946128	Mit outer
ATP2C1		19	0	0.01279461		Golgi
FKBP8	1	18	0.00165563	0.01212121	7.321212121	Mit outer
GOSR1		19	0	0.01279461		Golgi
EPHX1	1	18	0.00165563	0.01212121	7.321212121	ER
FAM114A2		18	0	0.01212121		Nucleus
SPAG9	1	17	0.00165563	0.01144781	6.914478114	Nucleus
TM9SF3	1	17	0.00165563	0.01144781	6.914478114	Others
SPTLC2		18	0	0.01212121		ER
EMC8	1	17	0.00165563	0.01144781	6.914478114	ER

ATP13A1		18	0	0.01212121		ER
CENPH		17	0	0.01144781		Nucleus
SEC24B	1	16	0.00165563	0.01077441	6.507744108	Others
MARCH5		17	0	0.01144781		ER Mit outer
GHDC	1	16	0.00165563	0.01077441	6.507744108	ER
ESYT2	1	16	0.00165563	0.01077441	6.507744108	ER
COMT		17	0	0.01144781		Others
ABHD12		17	0	0.01144781		Others
TMEM205		16	0	0.01077441		Others
MTCH2	1	15	0.00165563	0.01010101	6.101010101	Mit inner
DHRS7		16	0	0.01077441		Others
DHRS7		16	0	0.01077441		Others
SYAP1		15	0	0.01010101		Others
STIM1	1	14	0.00165563	0.00942761	5.694276094	ER
PEX19	1	13	0.00165563	0.00875421	5.287542088	Peroxisome
STXBP3		14	0	0.00942761		Others
STX5	1	13	0.00165563	0.00875421	5.287542088	Golgi
SOAT1		14	0	0.00942761		ER
TPD52L2	1	13	0.00165563	0.00875421	5.287542088	Others
OSBPL9	1	13	0.00165563	0.00875421	5.287542088	Golgi
EMC4		14	0	0.00942761		ER
AAAS		14	0	0.00942761		Nucleus
CAMLG		13	0	0.00875421		ER
NDC1		13	0	0.00875421		Nucleus
ATP6V0A1		13	0	0.00875421		Others
SURF4		13	0	0.00875421		ER
SLC27A4		13	0	0.00875421		ER
MCU		13	0	0.00875421		Mit inner
OSBPL8		13	0	0.00875421		ER
OCIAD1		13	0	0.00875421		Endosome
DIABLO		13	0	0.00875421		Mit outer
ATP6V0D1		13	0	0.00875421		Others
CHCHD6		12	0	0.00808081		Mit inner
ATP2B4		12	0	0.00808081		Others
QSOX2		12	0	0.00808081		Nucleus
MOSPD2		12	0	0.00808081		Others
SNAP29		12	0	0.00808081		Golgi
TDRKH		12	0	0.00808081		Mit outer
LNPBK		12	0	0.00808081		ER
NUP210		12	0	0.00808081		ER
MCL1		12	0	0.00808081		Mit outer
CDKAL1		12	0	0.00808081		ER