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Peer Review File

Molecular bases of the interactions of ATG16L1 with FIP200 and ATG8 family proteins

Corresponding Author: Professor Lifeng Pan

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
ATG16L1 is a component of the ATG12-ATG5-ATG16L1 E3 ligase complex for ATG8 (GABARAPs, LC3s) lipidation and plays a crucial role in autophagosome formation. It is known that ATG16L1 directly binds to FIP200, WIPI2 and ATG8s, essential factors for autophagosome formation; however, the molecular mechanism governing the specific interaction of ATG16L1 with FIP200 and ATG8s remains elusive. In this manuscript, the authors performed extensive biochemical analyses and revealed that ATG16L1 (235-247) is a functional FIP200 interacting region (FIR) motif that directly binds both FIP200 and ATG8s with different affinities for six ATG8 homologs. Moreover, the authors determined the crystal structure of the ATG16L1 LIR-FIP200 and ATG16LIR-GABARAPL1 complexes and unveiled the detailed interactions constructing these complexes. Importantly, the authors showed that ATG16L1 can form a ternary complex with FIP200 and WIPI2, but that ATG16L1-ATG8 interaction competes with ATG16L1-FIP200 and ATG16L1-WIPI2 interactions. Finally, the authors identified point mutations that specifically inhibit ATG16L1-FIP200 interaction and showed that this interaction is important for starvation-induced autophagy.
The extensive biochemical and structural analyses are solid and convincing, and the unveiled interactions of ATG16L1 with FIP200 and ATG8s are novel. The results obtained are not surprising, but they provide a very important molecular basis for the autophagy field, and the acquisition of an ATG16L1 mutant that only loses its interaction with FIP200 will contribute to furthering more precise functional analysis. Therefore, this reviewer considers that this manuscript worths publication. Only minor points are listed below.

Minor comments
1) In Fig. 5c,d, include the negative control cells (16KO without ATG16L1 expression) and compare and discuss the phenotypical difference between 16KO and mutant-expressing 16KO.
2) At lines 158-160, the authors wrote that “the hydrophobic side chain of ATG16L1 I240 deeply inserts into a hydrophobic pocket formed by the side chains of C1565, A1567, F1574 and F1582 residues of FIP200 Claw”. From Fig. 2c,d, it appears that the side chain of R1573 also contributes significantly to the construction of the pocket, so R1573 should be additionally listed.
3) At line 172, “Y1568A” should be “K1568A”.
4) The authors performed the titrations of 15N-labeled GABARAPL1 with unlabeled ATG16L1(235-247) or ATG16L1(78-247) in Supplementary Fig. 8. Because chemical shit assignment of GABARAPL1 is available at the Biological Magnetic Resonance Bank, quantify the chemical shift values and/or changes in signal intensities and map them on the GABARAPL1 model to confirm that the FIR binds to the canonical LIR-binding sites in GABARAPL1 in solution.

Reviewer #2

(Remarks to the Author)
Molecular bases of the interactions of ATG16L1 with FIP200 and ATG8 family proteins

The manuscript from Gong et al., details their findings characterising the interaction of FIP200 and ATG16L1 through their identification of a dual FIP200-interaction region (FIR) and Atg8-interaction motif(AIM). The authors detail the biochemical properties and interactions between the same FIR/AIM with both FIP200-claw domain and LC3/GABARAP proteins. The

authors provide crystal structures of the FIR/AIM with both FIP200-claw and GABARAP-L1. Finally, the authors identify mutations in the FIR/AIM sequence that abolished both ATG16L1-FIP200/ATG16L1-GABARAP-l1 interactions (IQIQ) and mutations that abolished fip200 but enhanced ATG8 interaction (DRIF).

The authors prove ample, solid biochemical evidence of the interaction between ATG16L1 and FIP200/Atg8 proteins. However, the manuscript in its current form is provides only an incremental gain on our knowledge of the mechanisms driving the interaction of FIP200 with components of the autophagy machinery and crosstalk with the Atg8 proteins. Due to previously publications by the corresponding author and others in the field
For example:

• FIP200 Claw Domain Binding to p62 Promotes Autophagosome Formation at Ubiquitin Condensates (2019)10.1016/j.molcel.2019.01.035. PDB: 6DCE

• Structural basis for TNIP1 binding to FIP200 during mitophagy
Wu, Shengmei et al. (2024). DOI: 10.1016/j.jbc.2024.107605. PDB: 8YFK

From the current authors lab:
• Zhou, Z., Liu, J., Fu, T. et al. Phosphorylation regulates the binding of autophagy receptors to FIP200 Claw domain for selective autophagy initiation
https://doi.org/10.1038/s41467-021-21874-1. (2021) PDB: 7CZM/7D0E

In its current form, the manuscript would fit well in a biochemical journal.

However, where the manuscript could be significantly improved and bring new knowledge to the field is more functional data on the consequences of these interactions and implications when they are disrupted. For this, the authors should focus on the role these interactions play in directing non-canonical lc3 lipidation on single membranes.

The manuscript and data needs more functional relevance to have a wider appeal and impact.

This is essential as the dual role ATG16L1 plays in both canonical and non-canonical autophagy, especially considering the interactions between FIP200 and WIPI2, which the authors detail, are dispensable for non-canonical/CASM mediated LC3 lipidation.

The authors need to combine their ATG16L1 mutants (IQIQ/DRIF) with K490A mutation of ATG16L1. The K490A mutation abolishes CASM function of ATG16L1 but does not affect canonical autophagy function of ATG16L1. Therefore, this mutation, in combination with the others, is essential for the authors to unpick the functional interactions they have shown. Indeed, the abolishment of FIR/LIR (IQIQ mutant) could prevent CASM LC3 conjugation.

The authors should use CASM specific inducers, such as monensin and/or nigericin to specifically induce CASM and BafA1 to inhibit CASM in the context of the ATG16L1 KO cells reconstituted with the mutants detailed above. This will allow the authors to specifically dress the role of the LC3/GABARAP interaction on LC3/GABARAP lipidation and autophagy flux.

The authors should show how the complexes (ATG16L1/FIP200/WIPI2 -ATG16L1/GABARAPL1 and ATG16/ATG5/ATG12 behave, through co-immunoprecipitations under both CASM and canonical (starvation) stimulation. Do the interactions between the components change depending on the stimuli? This is an essential experiment to show biologically relevant interactions. Where possible, endogenous interactions should be shown, or ATG16L1 reconstituted cells.

How is interaction between ATG16/5-12 complex affected by mutants (IQIQ/DRIF)?

In figure 5, the expression of ATG16L1 WT and mutants is vastly different. The authors need to have close to equal expression of WT and mutants to accurately assess the effects they are having on autophagy flux. With massively overexpressed mutants, it is difficult to compare effects.

The LC3 blots are difficult to interpret as they look overexposed. The authors should quantify LC3-II levels as well as p62 to assess the effect. BafA1 alone also needs to be included as a control as this will reveal any differences in basal autophagy flux.

The authors need to revise the model in figure 5e as its unclear and not supported by the evidence so far.

Reviewer #3

(Remarks to the Author)
Gong et al. characterise the molecular interactions of ATG16L1 with FIP200 and GABARAPL1. They presented high resolution structures of ATG16L1 FIR bound to FIP200 Claw domain and to GABARAPL1 and based on these structures they were able to design a mutant of ATG16L1 that can only bind GABARAPL1 but not FIP200 (ATG16L1 DRIF) and another ATG16L1 that cannot bind both GABARAPL1 and FIP200 (ATG16L1 IQIQ). Using these mutants, they went on to

look at functional relevance of FIP200-binding ability in ATG16L1 in regulating starvation-induced autophagy and concluded that FIP200-ATG16L1 interaction is indispensable for LC3B lipidation and degradation of autophagic substrate, p62. In general, the structural data is solid, extending our understanding at molecular level the previously reported FIP200-ATG16L1 interaction (PMC3589088). However, "the exact function of FIP200 for binding in autophagy remained unclear in previous studies" as mentioned by the authors does not seem to be much clearer in this study. The cellular data remains weak, and the model is not clearly presented. Please address my following comments to improve the manuscripts.
1. All the protein gels do not have molecular weight markers – please include them. Indicating what parts of proteins expressed, whether they are tagged or untagged on the figures and figure legends would help the readers to follow. The "sum" in Figure 1 was not clearly explained.
2. In Figure 2a-b, one of the FIP200 claw domain molecules have an extra a0 helix. Do all of the molecules in the crystal structure have this? Is this not present in the apo-form (PDB ID: 6DCE) simply due to the length difference?
3. It is useful to show the interactions via size exclusion chromatography and ITC-based method (Figures 3f, 4c-h). But it is important to show in vitro pulldown assays of WT and mutant versions of ATG16L1 to FIP200 or ATG8s to compare levels of binding and amounts of proteins tested on the same gel.
4. Instead of overexpressing everything in Figure 5a-b, can the authors show the binding of Flag-ATG16L1 (WT and mutants) to endogenous ATG8s and FIP200?
5. Lines 306-307 state that "both ATG16L1 DRIF and ATG16L1 IQIQ mutants only partially restored amino acid starvation-induced LC3B": It is not clear what comparison was made to lead to this conclusion? Did the authors compare the amount of LC3B-II or the ratio of LC3B-II/LC3B-I? The proper conclusions cannot be withdrawn without quantification and an appropriate negative control which is ATG16 KO by itself. In addition, it was only showed that ATG16L1 IQIQ mutant cannot bind GABARAPL1 (Figures 4, 5a-b) and assumed that they would not bind other ATG8s (lines 303-304). We do not know from the provided data whether this mutant cannot bind to all of the ATG8s – Please confirm this experimentally. Similarly, the ATG16L1 DRIF mutant is claimed to bind all ATG8s even in the abstract, but the binding was only shown for GABARAPL1. As can be observed in Figure 5c, there may be still a significant amount of LC3B lipidation accumulated with starvation and BafA1 (again we need a negative control here) indicating some potential significant level of autophagy occurring; maybe this mutant can still bind LC3B to some extent? Can the authors show the lipidation level for GABARAPL1?
To assess autophagy, it is more meaningful to compare the amount of p62 degraded rather than comparing the levels of p62 left after autophagy induction because the WT and ATG16L1 have different starting levels of p62 (Figure 5c-d). Nevertheless, this experiment has too much of ATG16L1 mutants expressed compared to the WT ATG16L1 and overexpression of ATG16L1 has previously shown to inhibit autophagy (PMC2366860). So the potential lower autophagy levels in cells rescued with the ATG16L1 mutants than those rescued with WT ATG16L1 could be due to their overexpression. It is therefore suggested that these experiments should be performed with the Flag-ATG16L1 constructs used in Figure 5a which were able to be expressed at equal expression and ATG16 KO cells should be included.
6. Can the authors explain the model more clearly? What roles do the FIP200/ATG16L1 binding and ATG16L1/ATG8s play during autophagosome biogenesis? What are the effects of losing each interaction and both interactions i.e. what defects are you expecting?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed all of my concerns.

Reviewer #2

(Remarks to the Author)
The authors have addressed all my main concerns and I am happy with their revision and for the manuscript to be considered for publication.

I would suggest that the authors include proper negative controls in their Immunoprecipitations for future studies. For example, Figure IV, VII should use GFP alone as a negive control for the IP.

The inclusion of functional data has improved the manuscript. The author could consider including Supp Fig 18 as part of figure 5 as this important distiction between cannonical and non-cannonical autophagy is worthy of inclusion in the manin figure.

Reviewer #3

(Remarks to the Author)
The authors have done a lot of work trying to address mine and other reviewers' comments. However, I think the evidence for the conclusion of the manuscript that "the interaction of ATG16L1 with FIP200 is indispensable for the effective autophagic flux" and the molecular significance "underlying the interactions of ATG16L1 with FIP200 and ATG8 family

proteins" remain unclear. Logically, it does not make sense that ATG16L1 IQIQ has less effect on p62 degradation than ATG16L1 DRIF (Fig 5d) while both mutants cannot bind FIP200 and can still bind endogenous GABARAP family in cells (Supp Fig 17). ATG16L1 IQIQ has still been referred to as a mutant that cannot bind both FIP200 and ATG8s throughout the manuscript but clearly this mutant is still capable of binding ATG8s in cells (Supp Fig 17). The difference of these two mutants in driving autophagy flux in p62 assay and LC3I/LC3II ratio assay also seems confusing because both assays should be showing autophagy/autophagy flux. In addition, in the blot (Fig 5c) p62 levels in ATG16L1 DRIF samples look very much different with the ATG16L1 KO samples, but the quantification (Fig 5d) shows no difference.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
The authors have sufficiently addressed my concerns. I now support the publication of the work.

Point-by-point responses to the reviewers' comments:
(Reviewers' comments are in **blue**, and our responses are in **black**)

Specific responses to the criticisms from the Reviewer #1:
We are very grateful to the strong supports and constructive suggestions from the reviewer. The following is our detailed responses to the reviewer's comments:

ATG16L1 is a component of the ATG12-ATG5-ATG16L1 E3 ligase complex for ATG8 (GABARAPs, LC3s) lipidation and plays a crucial role in autophagosome formation. It is known that ATG16L1 directly binds to FIP200, WIPI2 and ATG8s, essential factors for autophagosome formation; however, the molecular mechanism governing the specific interaction of ATG16L1 with FIP200 and ATG8s remains elusive. In this manuscript, the authors performed extensive biochemical analyses and revealed that ATG16L1 (235-247) is a functional FIP200 interacting region (FIR) motif that directly binds both FIP200 and ATG8s with different affinities for six ATG8 homologs. Moreover, the authors determined the crystal structure of the ATG16L1 LIR-FIP200 and ATG16LIR-GABARAPL1 complexes and unveiled the detailed interactions constructing these complexes. Importantly, the authors showed that ATG16L1 can form a ternary complex with FIP200 and WIPI2, but that ATG16L1-ATG8 interaction competes with ATG16L1-FIP200 and ATG16L1-WIPI2 interactions. Finally, the authors identified point mutations that specifically inhibit ATG16L1-FIP200 interaction and showed that this interaction is important for starvation-induced autophagy.

The extensive biochemical and structural analyses are solid and convincing, and the unveiled interactions of ATG16L1 with FIP200 and ATG8s are novel. The results obtained are not surprising, but they provide a very important molecular basis for the autophagy field, and the acquisition of an ATG16L1 mutant that only loses its interaction with FIP200 will contribute to furthering more precise functional analysis. Therefore, this reviewer considers that this manuscript worths publication. Only minor points are listed below.

Minor comments

1) In Fig. 5c,d, include the negative control cells (16KO without ATG16L1 expression) and compare and discuss the phenotypical difference between 16KO and mutant-expressing 16KO.
We understand the reviewer's concern. Following the reviewer's nice suggestions, we have added the negative control *ATG16L1* KO cell line to compare the p62 and LC3B-II levels in *ATG16L1* KO cell line and *ATG16L1* KO cell lines reconstituted with the wild type ATG16L1 or relevant ATG16L1 mutants (see **Fig. V** below, and **Fig. 5c**, **d**, **e** in the revised manuscript). Meanwhile, we have also discussed the phenotypical differences between *ATG16L1* KO cell line and *ATG16L1* KO cell lines reconstituted with the wild type ATG16L1 or relevant ATG16L1 mutants in the revised manuscript.

2) At lines 158-160, the authors wrote that "the hydrophobic side chain of ATG16L1 I240 deeply inserts into a hydrophobic pocket formed by the side chains of C1565,

A1567, F1574 and F1582 residues of FIP200 Claw". From Fig. 2c,d, it appears that the side chain of R1573 also contributes significantly to the construction of the pocket, so R1573 should be additionally listed.

We thank the reviewer for pointing out this. According to reviewer's comment, we have modified our statement and added the contribution of the R1573 residue of FIP200 Claw for interacting with ATG16L1 I240 in the revised manuscript.

3) At line 172, "Y1568A" should be "K1568A".

We are sorry for our carelessness, and thank the reviewer for pointing out this mistake in our writing. We have corrected this error in the revised manuscript.

4) The authors performed the titrations of 15N-labeled GABARAPL1 with unlabeled ATG16L1(235-247) or ATG16L1(78-247) in Supplementary Fig. 8. Because chemical shit assignment of GABARAPL1 is available at the Biological Magnetic Resonance Bank, quantify the chemical shift values and/or changes in signal intensities and map them on the GABARAPL1 model to confirm that the FIR binds to the canonical LIR-binding sites in GABARAPL1 in solution.

Following the reviewer's nice suggestion, based on the previous NMR assignment of GABARAPL1[1], we have assigned the NMR peaks in the ^{1}H-^{15}N HSQC spectrum of the ^{15}N-labeled GABARAPL1 and quantified the chemical shift changes in our NMR titration experiments (see **Fig. Ia** below, and **Supplemental Fig. 13a** in the revised manuscript). Moreover, we have mapped the chemical shift differences onto our crystal structure of the GABARAPL1/ATG16L1 FIR complex, revealing that the significant backbone amide chemical shift changes are only rich in ATG16L1 FIR-binding regions of GABARAPL1 (see **Fig. Ib** below, and **Supplemental Fig. 13b** in the revised manuscript), confirming that ATG16L1 FIR binds to the canonical AIM-binding sites of GABARAPL1 in solution.

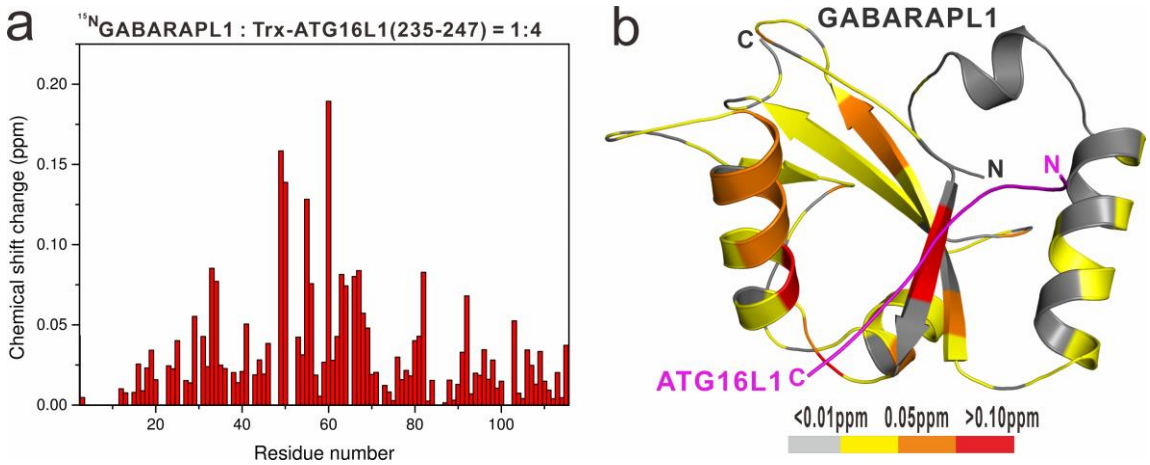

Figure I: NMR-based characterizations of the interaction between GABARAPL1 and ATG16L1 FIR. (**a**) Plot of backbone amide chemical shift differences and peak broadening as a function of the residue number of GABARAPL1 between the *apo*-form and the protein titrated with Trx-tagged ATG16L1(235-247) at a molar ratio of 1:4 in Supplementary Fig. 8a of the revised manuscript. In this representation, the combined ^{1}H and ^{15}N chemical shift changes are defined as:

$$\Delta_{ppm} = [(\Delta\delta_{HN})^2 + (\Delta\delta_N \times \alpha_N)^2]^{1/2}$$

where $\Delta\delta_{HN}$ and $\Delta\delta_{N}$ represent differences of the amide proton and nitrogen chemical shifts of each residue of GABARAPL1. The scaling factor (α_N) used to normalize the ^{1}H and ^{15}N chemical shifts is 0.142. (**b**) The ribbon representation shows the chemical shift changes of panel **a** mapped onto the crystal structure of the ATG16L1 FIR/GABARAPL1 complex.

Specific responses to the criticisms from the Reviewer #2:
We appreciate the reviewer's constructive criticisms and insightful suggestions to our manuscript, as these have been valuable in improving the scientific quality of the manuscript. The following is our detailed responses to the reviewer's comments:

The manuscript from Gong et al., details their findings characterising the interaction of FIP200 and ATG16L1 through their identification of a dual FIP200-interaction region (FIR) and Atg8-interaction motif (AIM). The authors detail the biochemical properties and interactions between the same FIR/AIM with both FIP200-claw domain and LC3/GABARAP proteins. The authors provide crystal structures of the FIR/AIM with both FIP200-claw and GABARAP-L1. Finally, the authors identify mutations in the FIR/AIM sequence that abolished both ATG16L1-FIP200/ATG16L1-GABARAP-l1 interactions (IQIQ) and mutations that abolished fip200 but enhanced ATG8 interaction (DRIF).

The authors prove ample, solid biochemical evidence of the interaction between ATG16L1 and FIP200/Atg8 proteins. However, the manuscript in its current form is provides only an incremental gain on our knowledge of the mechanisms driving the interaction of FIP200 with components of the autophagy machinery and crosstalk with the Atg8 proteins. Due to previously publications by the corresponding author and others in the field
For example:

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From the current authors lab:
• Zhou, Z., Liu, J., Fu, T. et al. Phosphorylation regulates the binding of autophagy receptors to FIP200 Claw domain for selective autophagy initiation
https://doi.org/10.1038/s41467-021-21874-1. (2021) PDB: 7CZM/7D0E

In its current form, the manuscript would fit well in a biochemical journal.

However, where the manuscript could be significantly improved and bring new knowledge to the field is more functional data on the consequences of these interactions and implications when they are disrupted. For this, the authors should focus on the role these interactions play in directing non-canonical lc3 lipidation on single membranes.

The manuscript and data needs more functional relevance to have a wider appeal and impact.

This is essential as the dual role ATG16L1 plays in both canonical and non-canonical autophagy, especially considering the interactions between FIP200 and WIPI2, which the authors detail, are dispensable for non-canonical/CASM mediated LC3 lipidation.

The authors need to combine their ATG16L1 mutants (IQIQ/DRIF) with K490A mutation of ATG16L1. The K490A mutation abolishes CASM function of ATG16L1 but does not affect canonical autophagy function of ATG16L1. Therefore, this mutation, in combination with the others, is essential for the authors to unpick the functional interactions they have shown. Indeed, the abolishment of FIR/LIR (IQIQ mutant) could prevent CASM LC3 conjugation.

The authors should use CASM specific inducers, such as monensin and/or nigericin to specifically induce CASM and BafA1 to inhibit CASM in the context of the ATG16L1 KO cells reconstituted with the mutants detailed above. This will allow the authors to specifically dress the role of the LC3/GABARAP interaction on LC3/GABARAP lipidation and autophagy flux.

We thank the reviewer for the above constructive comments. Following the reviewer's nice comments, we have rescued the *ATG16L1* KO cell line with additional ATG16L1 K490A mutant, ATG16L1 K490A/D239R/I240F mutant (K490A/DRIF), or ATG16L1 K490A/I240Q/I243Q mutant (K490A/IQIQ). As expected, the *ATG16L1* knockout and the K490A mutation of ATG16L1 essentially abolish or significantly attenuate the monensin-induced CASM (see **Fig. II** below, and **Supplemental Fig. 18** in the revised manuscript). Meanwhile, the ATG16L1 DRIF mutant significantly facilitates monensin-induced LC3B lipidation in comparison with the wild type ATG16L1 and the ATG16L1 IQIQ mutant, suggesting that the interaction of ATG16L1 FIR with FIP200 impedes CASM, while the interaction of ATG16L1 FIR with ATG8 family proteins promotes this process (see **Fig. II** below, and **Supplemental Fig. 18** in the revised manuscript). Due to the facilitation from ATG8 family protein is counteracted by the inhibition from FIP200, the wild type ATG16L1 and the ATG16L1 IQIQ mutant exhibited comparable monensin-induced LC3B lipidation, consistent with a previous report[2].

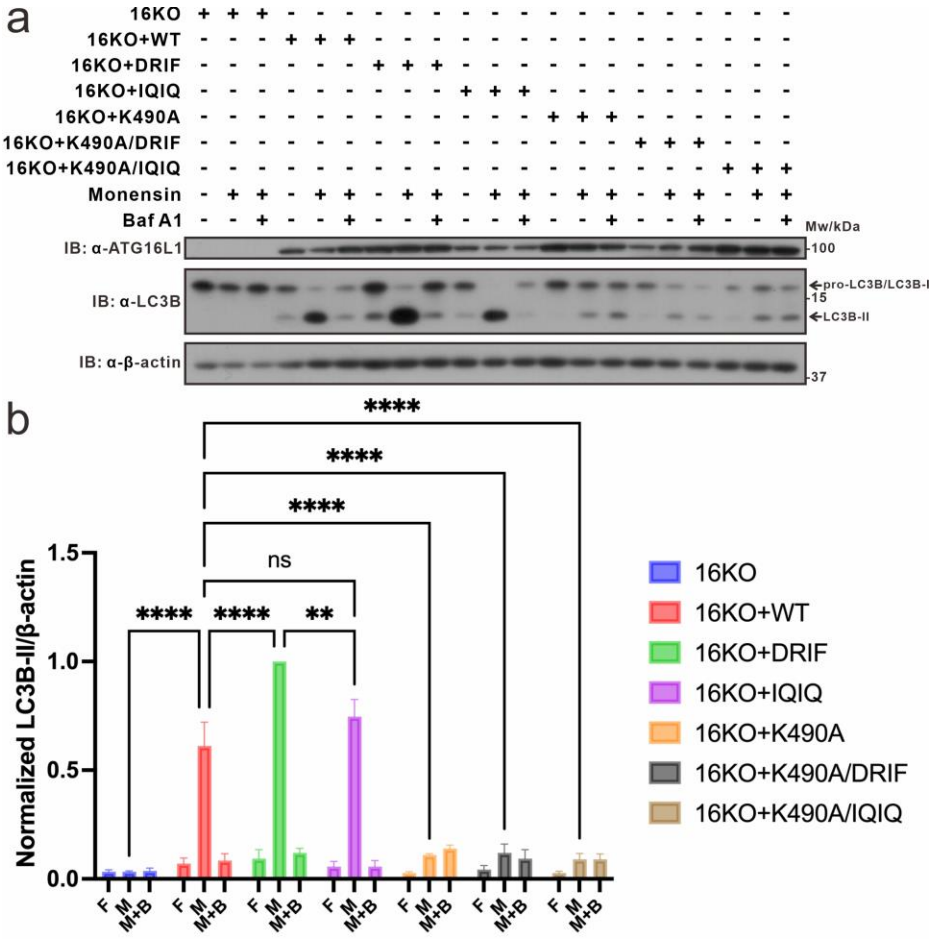

Figure II: Cell-based analyses of the monensin-induced CASM in the context of the *ATG16L1*-knockout cells reconstituted with relevant different ATG16L1 mutants. (**a**) Western blot-based measurements of the LC3B lipidation in *ATG16L1*-knockout HeLa cells (16KO) as well as that rescued with mEGFP-tagged WT ATG16L1 (16KO+WT), mEGFP-tagged ATG16L1 D239R/I240F mutant (16KO+DRIF), mEGFP-tagged ATG16L1 I240Q/I243Q mutant (16KO+IQIQ), mEGFP-tagged ATG16L1 K490A mutant (16KO+K490A), mEGFP-tagged ATG16L1 K490A/D239R/I240F mutant (16KO+K490A/DRIF), or mEGFP-tagged ATG16L1 K490A/I240Q/I243Q mutant (16KO+K490A/IQIQ) treated for 1 hour using D10+PS normal medium (F), D10+PS normal medium treated with monensin at 100 μM (M), or D10+PS normal medium treated with monensin at 100 μM and bafilomycin A1 at 400 nM (M+B). Cell lysates were immunoblotted for the indicated proteins. (**b**) The levels of LC3B-II and β-actin in panel **a** were quantified in ImageJ and normalized to that of 16KO+DRIF cells treated with monensin at 100 μM (M). The data is presented as means ± SEM from three independent experiments. Statistical analyses were performed in GraphPad Prism 9 by two-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons test, and P value style is P = 0.1234 [not significant (ns)], *P = 0.0332, **P = 0.0021, ***P = 0.0002, and ****P < 0.0001.

The authors should show how the complexes (ATG16L1/FIP200/WIPI2 - ATG16L1/GABARAPL1 and ATG16/ATG5/ATG12 behave, through co-

immunoprecipitations under both CASM and canonical (starvation) stimulation. Do the interactions between the components change depending on the stimuli? This is an essential experiment to show biologically relevant interactions. Where possible, endogenous interactions should be shown, or ATG16L1 reconstituted cells.

We thank the reviewer for these insightful and constructive comments. In response to the recommendations suggested by the reviewer, we have conducted relevant endogenous co-immunoprecipitation assays utilizing the *ATG16L1* KO cell line reconstituted with the GFP-tagged wild type ATG16L1. Due to the poor performances of the commercially available GABARAPL1 antibodies from both Proteintech (83817-1-RR) and Cell Signaling Technology (26632S), we were compelled to use a GABARAP family antibody from Selleck (F1156) as an alternative. Our co-immunoprecipitation results showed that the interactions of the GFP-tagged wild type ATG16L1 with endogenous ATG5, WIPI2 and GABARAP family proteins are almost not influenced by monensin or starvation stimulation (see **Fig. III** below, and **Supplemental Fig. 19** in the revised manuscript). However, the monensin treatment considerably impairs the ATG16L1/FIP200 binding (see **Fig. III** below, and **Supplemental Fig. 19** in the revised manuscript), in line with previous reports that FIP200 is not involved in the CASM process[3,4].

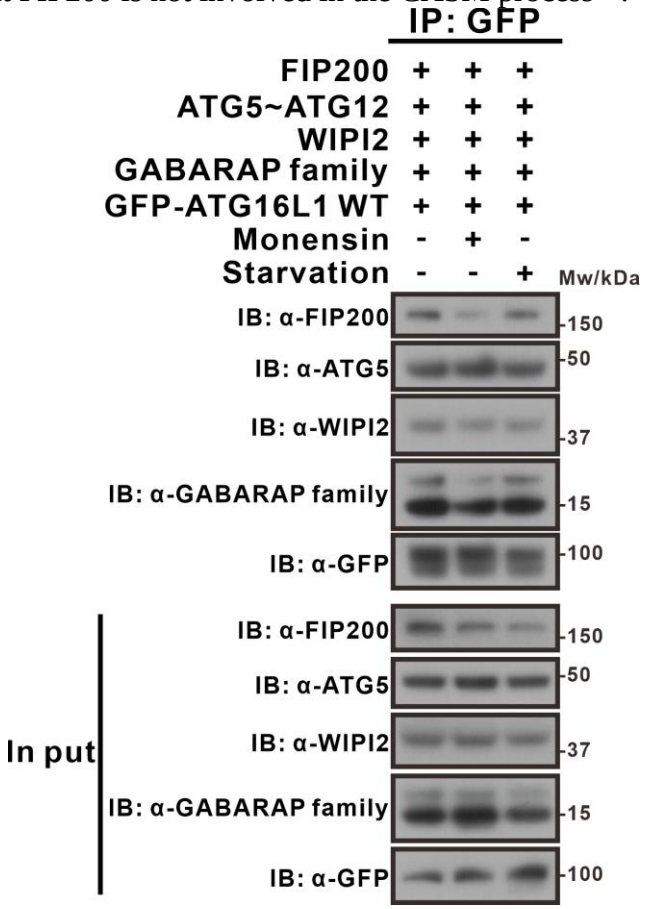

Figure III: Co-immunoprecipitation analyses of the interactions of the wild type ATG16L1 with the endogenous FIP200, ATG5, WIPI2 and GABARAP family proteins in response to monensin or starvation treatment. Co-immunoprecipitation assays showing that the interactions of the mEGFP-tagged wild type ATG16L1 with endogenous FIP200, ATG5, WIPI2 and GABARAP family proteins in response to

monensin or starvation treatment. “IB” means immunoblotting. “Monensin” means that cells are treated with monensin at 100 μM for 1 hour. “Starvation” means that cells are treated with amino acid starvation medium for 4 hours.

How is interaction between ATG16/5-12 complex affected by mutants (IQIQ/DRIF)?

Following the reviewer’s comment, we have performed relevant co-immunoprecipitation assays and revealed that the interaction between ATG5 and ATG16L1 is essentially not influenced by the ATG16L1 DRIF or IQIQ mutation (see **Fig. IV** below, and **Supplemental Fig. 17c** in the revised manuscript), consistent with a previous report that the N-terminal ATG5BD domain of ATG16L1 is responsible for associating with ATG5~ATG12[5].

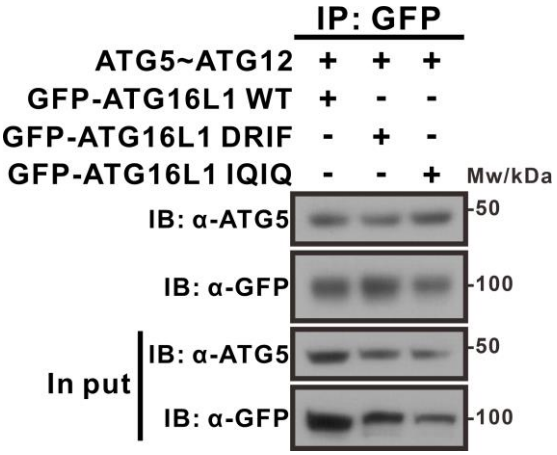

Figure IV: Co-immunoprecipitation analyses of the interaction of endogenous ATG5 with the wild type ATG16L1 or relevant ATG16L1 mutants. Co-immunoprecipitation assays showing that the interaction of endogenous ATG5 with the mEGFP-tagged wild type ATG16L1 (WT), ATG16L1 D239R/I240F (DRIF) mutant or I240Q/I243Q (IQIQ) mutant. “IB” means immunoblotting.

In figure 5, the expression of ATG16L1 WT and mutants is vastly different. The authors need to have close to equal expression of WT and mutants to accurately assess the effects they are having on autophagy flux. With massively overexpressed mutants, it is difficult to compare effects.

We understand the reviewer’s concern, and thank the reviewer for the above constructive comments. According to the reviewer’s suggestion, we have re-rescued the *ATG16L1* KO cell line with the wild type ATG16L1 or relevant ATG16L1 mutants. This time, the expression levels of ATG16L1 WT and mutants are comparable (see **Fig. Va** below, and **Fig. 5c** in the revised manuscript). Unsurprisingly, the autophagic flux in the *ATG16L1* knockout cells was effectively rescued by the wild-type ATG16L1. However, both ATG16L1 DRIF and ATG16L1 IQIQ mutants significantly impair the autophagic degradation of p62, underscoring an indispensable role of ATG16L1 FIR in starvation-induced autophagy (see **Fig. Va**, **b** below, and **Fig. 5c**, **d** in the revised manuscript). Notably, the ATG16L1 DRIF mutation induces a much more severe impediment of the p62 degradation than the ATG16L1 IQIQ mutation (see **Fig. Va**, **b** below, and **Fig. 5c**, **d** in the revised manuscript), implying that the interaction of ATG16L1 FIR with FIP200 facilitates the autophagy flux, whereas the interactions of ATG16L1 FIR with ATG8

family proteins attenuate the autophagy flux.

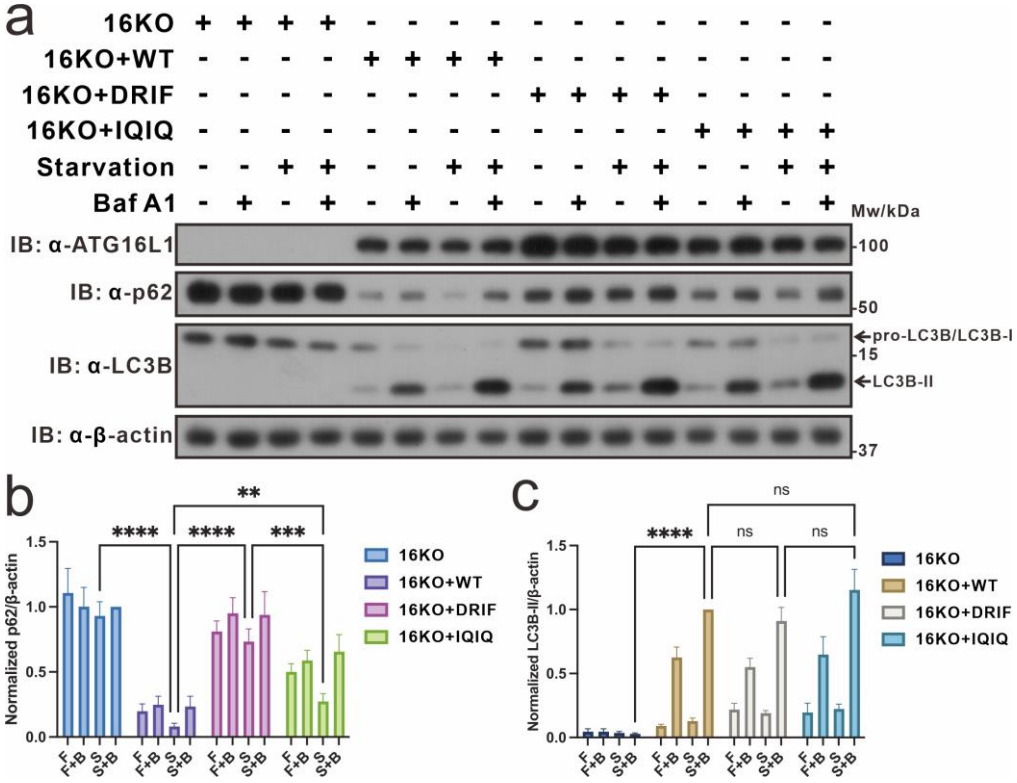

Figure V: Cell-based assays of the interactions of ATG16L1 with GABARAPL1 and FIP200 in starvation-induced autophagy. (**a**) Western blot-based measurements of the LC3B lipidation and p62 degradation levels in ATG16L1 knockout HeLa cells (16KO) as well as that rescued with mEGFP-tagged WT ATG16L1 (16KO+WT), mEGFP-tagged ATG16L1 D239R/I240F mutant (16KO+DRIF), or mEGFP-tagged ATG16L1 I240Q/I243Q mutant (16KO+IQIQ) treated for 4 hours using D10+PS normal medium (F), D10+PS normal medium treated with bafilomycin A1 at 400 nM (F+B), amino acid starvation medium (S), or amino acid starvation medium treated with bafilomycin A1 at 400 nM (S+B). Cell lysates were immunoblotted for the indicated proteins. (**b**) The levels of p62 and β-actin in panel **a** were quantified in ImageJ and normalized to that of 16KO cells treated with S+B. The data is presented as means ± SEM from four independent experiments. (**c**) The levels of LC3B-II and β-actin in in panel **a** were quantified in ImageJ and normalized to the 16KO+WT cells treated with S+B. The data is presented as means ± SEM from four independent experiments. Statistical analyses were both performed in GraphPad Prism 9 by two-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons test, and P value style is P = 0.1234 (not significant (ns)), *P = 0.0332, **P = 0.0021, ***P = 0.0002, and ****P < 0.0001.

The LC3 blots are difficult to interpret as they look overexposed. The authors should quantify LC3-II levels as well as p62 to assess the effect. BafA1 alone also needs to be included as a control as this will reveal any differences in basal autophagy flux.

Following the reviewer's nice comments, we have quantified the LC3B-II levels and included the BafA1 alone as a control (see **Fig. Va**, **c** above, and **Fig. 5c**, **e** in the revised manuscript). Our new results showed that the starvation-induced lipidation of LC3B is

unperturbed by the ATG16L1 DRIF or IQIQ mutation (see **Fig. Va**, **c** above, and **Fig. 5c**, **e** in the revised manuscript).

The authors need to revise the model in figure 5e as its unclear and not supported by the evidence so far.

Following the reviewer's comment, we have modified and updated the final model in the revised manuscript (see **Fig. 5f** in the revised manuscript). Based on our new results, we have concluded that the interaction of ATG16L1 FIR with FIP200 creates a positive feedback loop between WIPI2, ATG16L1 and FIP200 to facilitate the autophagy flux, whereas the interactions of ATG16L1 FIR with ATG8 family proteins impair this positive feedback loop to prevent the excessive activation of autophagy.

Specific responses to the criticisms from the Reviewer #3:

We thank the reviewer for the strong supports and enthusiastic comments of our manuscript. The following is a list of our detailed responses to the comments raised:

Gong et al. characterise the molecular interactions of ATG16L1 with FIP200 and GABARAPL1. They presented high resolution structures of ATG16L1 FIR bound to FIP200 Claw domain and to GABARAPL1 and based on these structures they were able to design a mutant of ATG16L1 that can only bind GABARAPL1 but not FIP200 (ATG16L1 DRIF) and another ATG16L1 that cannot bind both GABARAPL1 and FIP200 (ATG16L1 IQIQ). Using these mutants, they went on to look at functional relevance of FIP200-binding ability in ATG16L1 in regulating starvation-induced autophagy and concluded that FIP200-ATG16L1 interaction is indispensable for LC3B lipidation and degradation of autophagic substrate, p62. In general, the structural data is solid, extending our understanding at molecular level the previously reported FIP200-ATG16L1 interaction (PMC3589088). However, "the exact function of FIP200 for binding in autophagy remained unclear in previous studies" as mentioned by the authors does not seem to be much clearer in this study. The cellular data remains weak, and the model is not clearly presented. Please address my following comments to improve the manuscripts.

1. All the protein gels do not have molecular weight markers-please include them. Indicating what parts of proteins expressed, whether they are tagged or untagged on the figures and figure legends would help the readers to follow. The "sum" in Figure 1 was not clearly explained.

We are sorry for not providing the molecular weight markers in our protein gels in the original manuscript. Following the reviewer's nice comments, we have included relevant molecular weight markers and added the tag information in all our protein gels in the revised manuscript. Actually, the "Sum" in Figure 1 stands for the theoretical sum of the Trx-tagged ATG16L1 fragment and FIP200 Claw SEC profiles, while "Mixture" stands for the Trx-tagged ATG16L1 fragment and FIP200 Claw mixture sample. We have also added this information in the related figure legends in the revised manuscript.

2. In Figure 2a-b, one of the FIP200 claw domain molecules have an extra a0 helix. Do all of the molecules in the crystal structure have this? Is this not present in the apo-form (PDB ID: 6DCE) simply due to the length difference?

In the crystal structure of the ATG16L1 FIR/FIP200 Claw complex determined in this study, there are two ATG16L1 FIR molecules and two monomeric FIP200 Claw in an asymmetric unit, and only one monomeric FIP200 Claw has an extra α0 helix (see **Supplemental Fig. 2a** in the revised manuscript). Meanwhile, this extra α0 helix is also not observed in other reported crystal structures related to FIP200 Claw, such as the crystal structures of the FIP200 Claw/p-CCPG1 FIR2 complex (PDB ID: 7D0E) and the FIP200 Claw/p-Optineurin LIR complex (PDB ID: 7CZM). In fact, the space group of the FIP200 Claw/ATG16L1 FIR structure is $P12_11$ and different from that of the aforementioned structures, such as $P4_32_12$ for the FIP200 Claw/p-CCPG1 FIR2 complex (PDB ID: 7D0E) and $C121$ for the FIP200 Claw/p-Optineurin LIR complex (PDB ID: 7CZM). Thus, the extra α0 helix is attributed to the unique crystal packing mode of the FIP200 Claw/ATG16L1 FIR complex.

3. It is useful to show the interactions via size exclusion chromatography and ITC-based method (Figures 3f, 4c-h). But it is important to show in vitro pulldown assays of WT and mutant versions of ATG16L1 to FIP200 or ATG8s to compare levels of binding and amounts of proteins tested on the same gel.

Following the reviewer's comments, we have constructed and purified the relevant GST-tagged proteins for the wild type ATG16L1 FIR (ATG16L1 FIR WT), the ATG16L1 FIR DRIF mutant and the ATG16L1 FIR IQIQ mutant. Further GST-pull down assays showed that FIP200 Claw interacts well with ATG16L1 FIR WT but not the ATG16L1 FIR DRIF and IQIQ mutants (see **Fig. VIa** below, and **Supplemental Fig. 15a** in the revised manuscript), in line with our size exclusion chromatography results. In parallel, our GST-pull down results of ATG16L1 FIR with ATG8 family proteins revealed that the bindings of ATG16L1 FIR to ATG8 family proteins are generally enhanced by the DRIF mutation but significantly attenuated or essentially abolished by the IQIQ mutation of ATG16L1 FIR (see **Fig. VIb**, **c** below, and **Supplemental Fig. 15b**, **c** in the revised manuscript). Meanwhile, due to the weak and dynamic interaction of ATG16L1 FIR with LC3B or GABARAPL2, we are unable to detect the effects of the DRIF mutation of ATG16L1 FIR on the interaction of ATG16L1 FIR with GABARAPL2 and the IQIQ mutation of ATG16L1 FIR on the interaction of ATG16L1 FIR with LC3B or GABARAPL2 (see **Fig. VIb**, **c** below, and **Supplemental Fig. 15b**, **c** in the revised manuscript). It is worthy to mention that as the GST-pull down assay is not a quantitative biochemical assay, the observed enhanced bindings for the ATG16L1 FIR DRIF mutant with relevant ATG8 family proteins may result from the alterations of k_{on} (the rate constant of association) and k_{off} (the rate constant of dissociation) values induced by the DRIF mutation of ATG16L1 FIR.

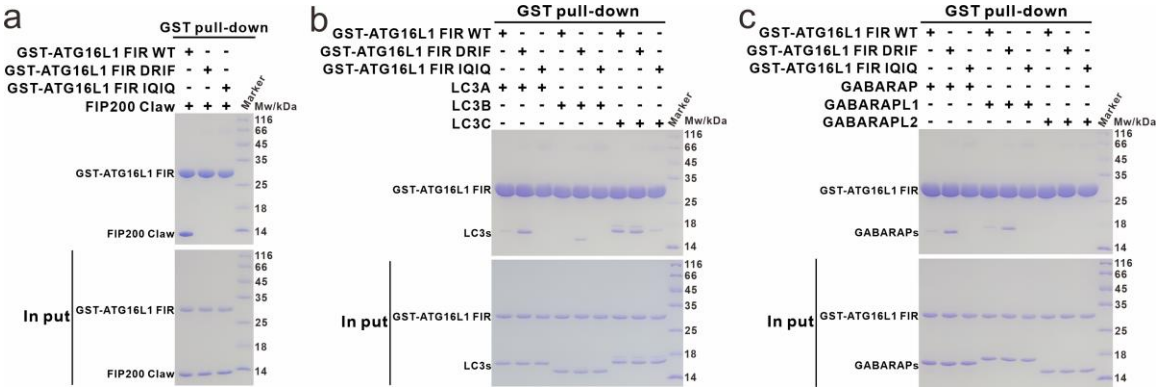

Figure VI: GST pull-down analyses of the interactions of the wild type ATG16L1 FIR or relevant mutants with FIP200 Claw and ATG8 family proteins. (**a**) GST pull-down assays of the interactions of FIP200 Claw with the GST-tagged wild type ATG16L1 FIR (ATG16L1 FIR WT), ATG16L1 D239R/I240F (DRIF) mutant and I240Q/I243Q (IQIQ) mutant. (**b**) GST pull-down assays of the interactions of ATG8 family proteins LC3A, LC3B, and LC3C with the GST-tagged wild type ATG16L1 FIR (ATG16L1 FIR WT), ATG16L1 D239R/I240F (DRIF) mutant and I240Q/I243Q (IQIQ) mutant. (**c**) GST pull-down assays of the interactions of ATG8 family proteins GABARAP, GABARAPL1, and GABARAPL2 with the GST-tagged wild type ATG16L1 FIR (ATG16L1 FIR WT), ATG16L1 D239R/I240F (DRIF) mutant and I240Q/I243Q (IQIQ) mutant.

4. Instead of overexpressing everything in Figure 5a-b, can the authors show the binding of Flag-ATG16L1 (WT and mutants) to endogenous ATG8s and FIP200?

Following the reviewer's constructive comment, we have conducted co-immunoprecipitation assays to measure the potential interactions of relevant ATG16L1 variants with endogenous FIP200 and GABARAP family proteins. Our Co-IP results showed that the wild type ATG16L1 but not the DRIF and IQIQ mutants of ATG16L1 can readily interact with endogenous FIP200 (see **Fig. VIIa** below, and **Supplemental Fig. 17a** in the revised manuscript). Unexpectedly, the wild type ATG16L1 and the ATG16L1 DRIF and IQIQ mutants exhibit a comparable capacity for binding to endogenous GABARAP family proteins (see **Fig. VIIb** below, and **Supplemental Fig. 17b** in the revised manuscript), suggesting that in addition to the direct ATG16L1 FIR/GABARAP family proteins interaction, ATG16L1 may also indirectly associate with GABARAP family proteins through other adaptors, such as the ATG5~ATG12/ATG3 complex as reported in previous studies[5-7].

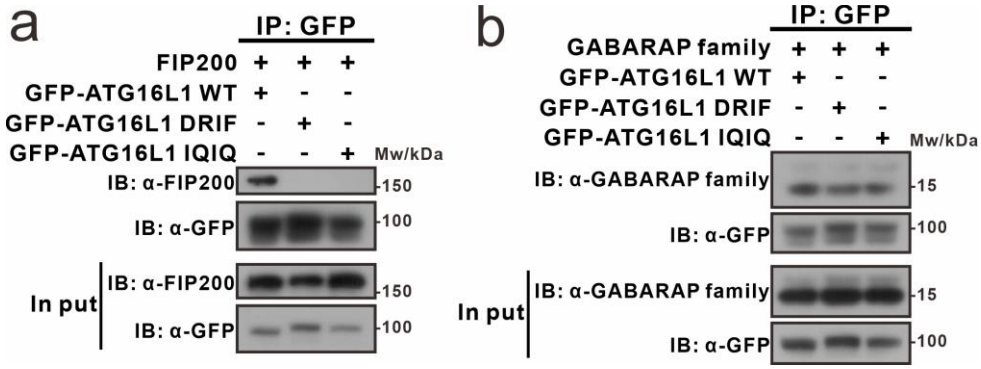

Figure VII: Co-immunoprecipitation analyses of the interactions of ATG16L1

variants with endogenous FIP200 and GABARAP family proteins. (**a** and **b**) Co-immunoprecipitation assays to measure the interactions of the mEGFP-tagged wild type ATG16L1 (WT), ATG16L1 D239R/I240F (DRIF) mutant and ATG16L1 I240Q/I243Q (IQIQ) mutant with endogenous FIP200 (**a**), or GABARAP family proteins (**b**). "IB" means immunoblotting.

5. Lines 306-307 state that "both ATG16L1 DRIF and ATG16L1 IQIQ mutants only partially restored amino acid starvation-induced LC3B": It is not clear what comparison was made to lead to this conclusion? Did the authors compare the amount of LC3B-II or the ratio of LC3B-II/LC3B-I? The proper conclusions cannot be withdrawn without quantification and an appropriate negative control which is ATG16 KO by itself. In addition, it was only showed that ATG16L1 IQIQ mutant cannot bind GABARAPL1 (Figures 4, 5a-b) and assumed that they would not bind other ATG8s (lines 303-304). We do not know from the provided data whether this mutant cannot bind to all of the ATG8s – Please confirm this experimentally. Similarly, the ATG16L1 DRIF mutant is claimed to bind all ATG8s even in the abstract, but the binding was only shown for GABARAPL1. As can be observed in Figure 5c, there may be still a significant amount of LC3B lipidation accumulated with starvation and BafA1 (again we need a negative control here) indicating some potential significant level of autophagy occurring; maybe this mutant can still bind LC3B to some extent? Can the authors show the lipidation level for GABARAPL1?
To assess autophagy, it is more meaningful to compare the amount of p62 degraded rather than comparing the levels of p62 left after autophagy induction because the WT and ATG16L1 have different starting levels of p62 (Figure 5c-d). Nevertheless, this experiment has too much of ATG16L1 mutants expressed compared to the WT ATG16L1 and overexpression of ATG16L1 has previously shown to inhibit autophagy (PMC2366860). So the potential lower autophagy levels in cells rescued with the ATG16L1 mutants than those rescued with WT ATG16L1 could be due to their overexpression. It is therefore suggested that these experiments should be performed with the Flag-ATG16L1 constructs used in Figure 5a which were able to be expressed at equal expression and ATG16 KO cells should be included.
We understand the reviewer's concerns, and thank the reviewer for the above constructive comments. Following the reviewer's suggestion, we have added the control *ATG16L1* KO cell line and quantitatively compare the LC3B-II levels in *ATG16L1* KO cell line and *ATG16L1* KO cell line reconstituted with the wild type ATG16L1 or relevant ATG16L1 mutant (see **Fig. Va**, **c** above, and **Fig. 5c**, **e** in the revised manuscript). See our detailed response to the point #5 from the reviewer 2. In addition, the expression levels of our re-rescued ATG16L1 WT and mutants in *ATG16L1* KO cell lines are comparable (see **Fig. Va** above, and **Fig. 5c** in the revised manuscript), thus allowing us to exclude the possibility that the ATG16L1 overexpression resulted in the inhibition of autophagy. Meanwhile, our new GST pull-down results showed that the ATG16L1 FIR DRIF mutant can bind almost all ATG8 family proteins, and the IQIQ mutation of ATG16L1 FIR significantly attenuated or essentially abolished the interactions between ATG16L1 FIR and ATG8 family proteins (see **Fig. VI** above, and **Supplemental Fig. 15** in the revised manuscript). Unfortunately, due to the poor performance of the GABARAPL1 antibody from both Proteintech (83817-1-RR) and Cell Signaling Technology (26632S), we are

unable to find a good GABARAPL1 antibody to detect the lipidation level of GABARAPL1.

6. Can the authors explain the model more clearly? What roles do the FIP200/ATG16L1 binding and ATG16L1/ATG8s play during autophagosome biogenesis? What are the effects of losing each interaction and both interactions i.e. what defects are you expecting?
Based on our new results, the loss of the FIP200/ATG16L1 binding resulted in the severe block of starvation-induced p62 degradation (see **Fig. Va**, **b** above, and **Fig. 5c**, **d** in the revised manuscript), suggesting that the FIP200/ATG16L1 binding facilitates the autophagic flux in starvation-induced autophagy. Despite the lack of a specific ATG16L1 mutant, which selectively binds to FIP200 but not ATG8 family proteins, the loss of both FIP200/ATG16L1 and ATG8s/ATG16L1 binding resulted in the alleviation of the block of p62 degradation due to the further loss of the ATG8s/ATG16L1 binding (see **Fig. Va, b** above, and **Fig. 5c**, **d** in the revised manuscript), implying that the ATG8s/ATG16L1 binding actually inhibits the autophagic flux in starvation-induced autophagy, in line with our biochemical observations that ATG8 family protein, such as GABARAPL1, directly competes with FIP200 and WIPI2 for binding to ATG16L1(207-247) (see **Fig. 4a**, **b** and **Supplemental Fig. 14b** in the revised manuscript). All in all, we concluded that the FIP200/ATG16L1 interaction generates a positive feedback loop to amplify the autophagic flux in starvation-induced autophagy, and then the abundant lipidated ATG8 family proteins can in turn compete with FIP200 for binding to ATG16L1 to attenuate this positive feedback loop, thereby preventing the excessive activation of autophagy. Following the reviewer's nice comment, we have modified the final model, and explain it more clearly in the revised manuscript (see **Fig. VIII** below, and **Fig. 5f** in the revised manuscript).

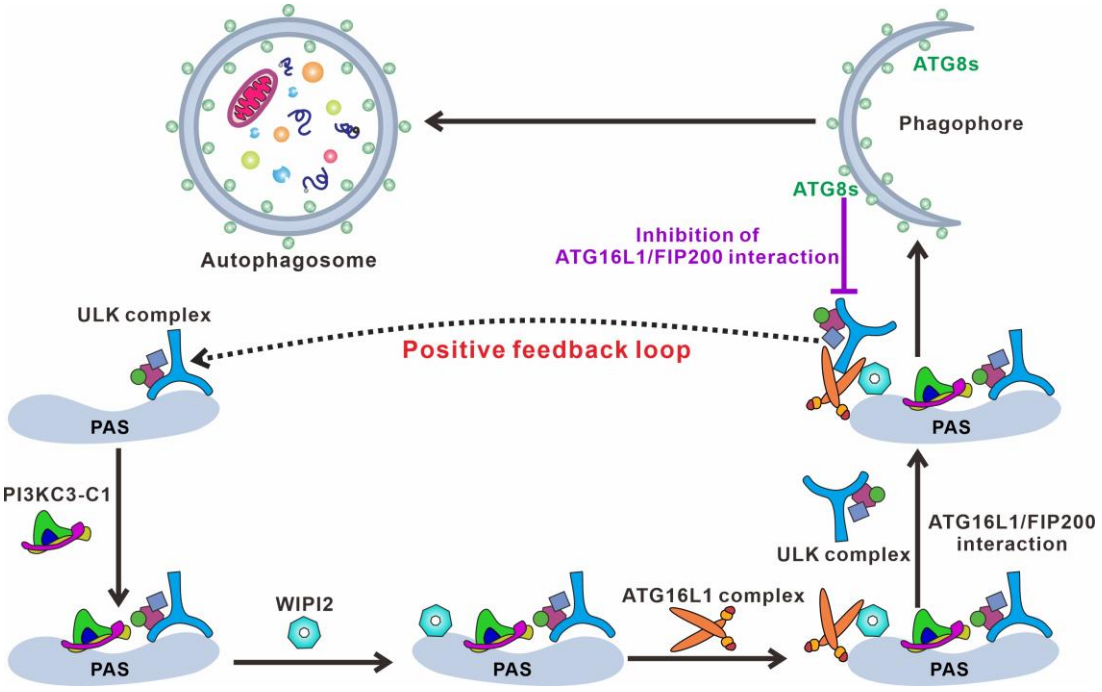

Figure VIII: A proposed model depicting the functions of the FIP200/ATG16L1 and ATG8s/ATG16L1 interactions in canonical autophagy. In this model, the specific FIP200/ATG16L1 interaction creates a positive feedback loop to amplify the autophagic

flux in starvation-induced autophagy, while the abundant lipidated ATG8 family proteins can attenuate this positive feedback loop by competing with FIP200 for binding to ATG16L1, thereby preventing the excessive activation of autophagy.

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1. Rozenknop, A. et al. Characterization of the interaction of GABARAPL-1 with the LIR motif of NBR1. *J Mol Biol* **410**, 477-87 (2011).
2. Fletcher, K. et al. The WD40 domain of ATG16L1 is required for its non-canonical role in lipidation of LC3 at single membranes. *EMBO J* **37**(2018).
3. Xu, Y. et al. A Bacterial Effector Reveals the V-ATPase-ATG16L1 Axis that Initiates Xenophagy. *Cell* **178**, 552-566 e20 (2019).
4. Fischer, T.D., Wang, C., Padman, B.S., Lazarou, M. & Youle, R.J. STING induces LC3B lipidation onto single-membrane vesicles via the V-ATPase and ATG16L1-WD40 domain. *J Cell Biol* **219**(2020).
5. Otomo, C., Metlagel, Z., Takaesu, G. & Otomo, T. Structure of the human ATG12~ATG5 conjugate required for LC3 lipidation in autophagy. *Nat Struct Mol Biol* **20**, 59-66 (2013).
6. Metlagel, Z., Otomo, C., Takaesu, G. & Otomo, T. Structural basis of ATG3 recognition by the autophagic ubiquitin-like protein ATG12. *Proc Natl Acad Sci U S A* **110**, 18844-9 (2013).
7. Farnung, J. et al. Semisynthetic LC3 Probes for Autophagy Pathways Reveal a Noncanonical LC3 Interacting Region Motif Crucial for the Enzymatic Activity of Human ATG3. *ACS Cent Sci* **9**, 1025-1034 (2023).

Point-by-point responses to the reviewers' comments:
(Reviewers' comments are in **blue**, and our responses are in **black**)

Specific responses to the criticisms from the Reviewer #1:
The authors have addressed all of my concerns.
We thank the reviewer for the strong support of our manuscript.

Specific responses to the criticisms from the Reviewer #2:
The authors have addressed all my main concerns and I am happy with their revision and for the manuscript to be considered for publication.

I would suggest that the authors include proper negative controls in their Immunoprecipitations for future studies. For example, Figure IV, VII should use GFP alone as a negive control for the IP.

The inclusion of functional data has improved the manuscript. The author could consider including Supp Fig 18 as part of figure 5 as this important distiction between cannonical and non-cannonical autophagy is worthy of inclusion in the manin figure.
We thank the reviewer for the above constructive comment. Following the reviewer's nice suggestion, we have included the original Supplementary Fig. 18 as a part of Fig. 5 in the revised manuscript (see **Fig. 5f, g** in the revised manuscript).

Specific responses to the criticisms from the Reviewer #3:
The authors have done a lot of work trying to address mine and other reviewers' comments. However, I think the evidence for the conclusion of the manuscript that "the interaction of ATG16L1 with FIP200 is indispensable for the effective autophagic flux" and the molecular significance "underlying the interactions of ATG16L1 with FIP200 and ATG8 family proteins" remain unclear. Logically, it does not make sense that ATG16L1 IQIQ has less effect on p62 degradation than ATG16L1 DRIF (Fig 5d) while both mutants cannot bind FIP200 and can still bind endogenous GABARAP family in cells (Supp Fig 17). ATG16L1 IQIQ has still been referred to as a mutant that cannot bind both FIP200 and ATG8s throughout the manuscript but clearly this mutant is still capable of binding ATG8s in cells (Supp Fig 17). The difference of these two mutants in driving autophagy flux in p62 assay and LC3I/LC3II ratio assay also seems confusing because both assays should be showing autophagy/autophagy flux. In addition, in the blot (Fig 5c) p62 levels in ATG16L1 DRIF samples look very much different with the ATG16L1 KO samples, but the quantification (Fig 5d) shows no difference.
We thank the reviewer for these insightful and constructive criticisms to our manuscript, as these have been valuable in improving the scientific quality of the manuscript. In fact, the cellular associations of ATG16L1 with ATG8 family proteins can be mediated by two different approaches, the ATG16L1 ATG5BD-mediated indirect interaction upon the formation of the ATG16L1-ATG5~ATG12/ATG3 complex to catalyze the the lipidation of ATG8 family proteins[1-3], and the ATG16L1 FIR-mediated direct interaction that inhibits the ATG16L1/FIP200 interaction-involved autophagy process. Due to the indirect and relatively stronger interaction between ATG16L1 ATG5BD and the ATG5~ATG12/ATG3 complex[1-3], the sole disruption of the direct association of

ATG16L1 FIR with ATG8 family proteins cannot strikingly reduce the associations of the full-length ATG16L1 with ATG8 family proteins in cells. Therefore, we wondered whether we could remove the ATG5BD region of ATG16L1 to avoid the potential influence of the indirect interaction of ATG16L1 with ATG8 family proteins. To this end, we have constructed relevant ATG16L1(78-607) variants and conducted a Co-IP assay to measure their interactions with endogenous GABARAP family proteins in the *ATG16L1*-knockout cell line. Our Co-IP results showed that the wild type ATG16L1(78-607) and the ATG16L1(78-607) DRIF mutant but not the IQIQ mutant of ATG16L1(78-607) can readily interact with endogenous GABARAP family proteins (see **Fig. I** below, and **Supplemental Fig. 17d** in the revised manuscript). Therefore, the ATG16L1 IQIQ mutant cannot directly interact with ATG8 family proteins in cells. As for the difference of these ATG16L1 DRIF and IQIQ mutants in driving autophagy flux in p62 assay and LC3I/LC3II ratio assay, actually both the p62 degradation level and the LC3I/LC3II ratio in general can reflect autophagy flux. However, the interaction between FIP200 and ATG16L1 actually directs the ATG16L1 complex to the pre-autophagosomal structure where generates autophagy flux[4]. Consequently, the lipidation of ATG8 family proteins catalyzed by the ATG16L1 complex containing the ATG16L1 DRIF or IQIQ mutant localized in aberrant single membranes rather than the phagophore membrane owing to the disruption of ATG16L1 FIR/FIP200 interaction cannot represent the real autophagy flux. Finally, we are sorry for not providing the suitable representative p62 level results in the previous manuscript, and actually, the p62 levels in ATG16L1 DRIF samples are similar to the ATG16L1 KO samples as shown in the updated Fig. 5c in the revised manuscript (see **Fig. 5c** in the revised manuscript).

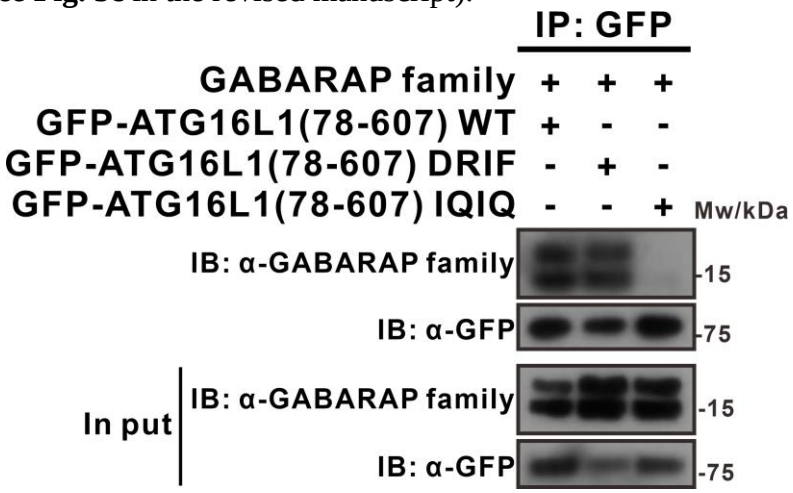

Fig. I: Co-immunoprecipitation analyses of the cellular interactions of relevant ATG16L1(78-607) variants with endogenous GABARAP family proteins. Co-immunoprecipitation assays showing that the endogenous interactions of endogenous GABARAP family proteins with the mEGFP-tagged wild type ATG16L1(78-607) (WT), ATG16L1(78-607) D239R/I240F (DRIF) mutant and ATG16L1(78-607) I240Q/I243Q (IQIQ) mutant. “IB” means immunoblotting.

References:

1. Metlagel, Z., Otomo, C., Takaesu, G. & Otomo, T. Structural basis of ATG3 recognition by the autophagic ubiquitin-like protein ATG12. *Proc Natl Acad Sci U S A* **110**, 18844-9 (2013).
2. Farnung, J. et al. Semisynthetic LC3 Probes for Autophagy Pathways Reveal a Noncanonical LC3 Interacting Region Motif Crucial for the Enzymatic Activity of Human ATG3. *ACS Cent Sci* **9**, 1025-1034 (2023).
3. Otomo, C., Metlagel, Z., Takaesu, G. & Otomo, T. Structure of the human ATG12~ATG5 conjugate required for LC3 lipidation in autophagy. *Nat Struct Mol Biol* **20**, 59-66 (2013).
4. Nishimura, T. et al. FIP200 regulates targeting of Atg16L1 to the isolation membrane. *EMBO Rep* **14**, 284-91 (2013).

Point-by-point responses to the comments raised by the referees
(Referees' comments are in **blue**, and our responses are in **black**)

Specific responses to the comments from the Reviewer #3:
The authors have sufficiently addressed my concerns. I now support the publication of the work.
We thank the reviewer for his/her strong supports of our manuscript.