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FIP200 regulates targeting of Atg16L1 to the isolation membrane

Taki Nishimura, Takeshi Kaizuka, Ken Cadwell, Mayurbhai Himatbhai Sahani, Tatsuya Saitoh, Shizuo Akira, Herbert W. Virgin and Noboru Mizushima

Corresponding author: Noboru Mizushima, The University of Tokyo, Graduate School and Faculty of Medicine

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 30 August 2012

Thank you for the submission of your manuscript to EMBO reports. I would like to apologize for not being able to reach a decision on your study sooner. We have now received the enclosed reports on it.

As you will see, all referees find the topic of your manuscript interesting but two referees feel that the data needs to be strengthened to be conclusive and suitable for publication.

In addition to general technical concerns raised by both referees #2 and #4 the following major concerns are noted. Referee #2 states that stronger evidence for the effects of the different Atg16 mutants on overall autophagic activity should be provided with additional assays. Referee #4 suggests that evidence showing that FIP200 must be associated with the ULK complex to bind Atg16L1 is needed to validate the model. In addition, this reviewer (and to some extent also referee #2) feels that in several other instances, further clarifications are needed in order to support the data at hand and to resolve what this referee considers to be inconsistencies with previous reports.

Overall, given these evaluations, the reviewers constructive comments and the potential interest of the study, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the referees (as outlined above and in their reports) must be addressed.

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Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions, except under exceptional circumstances in which a short extension is obtained from the editor. Also, the length may not exceed 27,500 characters (including spaces). Should you find the length constraints to be a problem, you may consider including any peripheral data in the form of Supplementary information (please include for review alongside the revised manuscript), to be published online only. However, materials and methods essential for the repetition of the key experiments should be described in the main body of the text and may not be displayed as supplemental information only.

We have also started encouraging authors to submit the raw data for western blots (i.e. original scans) to our editorial office. These data will be published online as part of the supplementary information. This is voluntary at the moment, but if you agree that this would be useful for readers I would like to invite you to supply these files when submitting the revised version of your study.

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We also welcome the submission of cover suggestions or motifs that might be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

REFEREE REPORTS:

Referee #2

Nishimura et al report on a new interaction between Atg16L1 and FIP200, the mammalian ortholog of Atg17. Furthermore, the authors identified the region on Atg16 essential for this interaction and examine their effect on protein localization. Accordingly, this interaction may be important in Atg16L1 recruitment to the isolation membrane.
Characterization of the early events of autophagosome biogenesis is of great importance. Identification of an interaction between Atg 16 and FIP200 is very interesting, however the data presented in this study are too preliminary to support the authors' conclusions. Most importantly, the authors fail to convincingly demonstrate an effect of the different Atg16 mutants on overall autophagic activity.

Specific comments:
1. Figure 1D, the input amounts of FIP200, Atg13 and Ulk1 are not equal in the different conditions. This may affect the authors' interpretation. In addition, their interaction with other complex subunits seems to decrease while the interaction with Atg16 increases following the DSP treatment. The authors need to address this issue.
The FIP200 band in figure 1F is too weak and unclear.
The authors should relate to the fact that FIP200 was not equally precipitated in all samples shown in Figure 1G. In contrast to the authors' conclusion, the ratio of FIP200 to Atg16 is different in the F/F vs. D/D samples.

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2. The experiment presented in Figure 2 supports reduction in individual protein but not complex stability directly as concluded by the authors. It is necessary to show complex formation by IP in the experiment presented in figure S2. It is also necessary to verify whether the decrease in protein stability is caused on the protein or mRNA level. Quantifications and statistical information should be performed.

3. The authors should relate to the fact that in figure 3B FLAG-tagged deletions and HA-FIP200 levels are not equal. This makes it difficult to conclude that there is a decrease in their interaction with FIP200.
In addition, the authors need to relate/describe all the results shown in figures 3B and C.
4. The authors do not relate to the WB describing p62 in the different samples in figure 4A. For example, it is not clear why p62 levels decrease in the presence of chloroquine.
The authors did not specify the exact conditions for the experiment described in figure 4B.
The authors need to explain why they chose to use of GFP-ULK1 in figures 4B and 5 instead of FIP200, the subject of the current study.
5. To support the model proposed by the authors it is necessary to determine the different Atg16 mutants and FIP200 KO effect on autophagy. This should be performed by different assays including for example long-lived protein degradation.

Referee #3

The authors have done an excellent job. The manuscript convincingly demonstrates that FIP200 regulates several events during the autophagosome formation.
Minor point:
Figure 4 panel A. It is odd to observe that p62 does not accumulate in the presence of chloroquine in lanes 3,9 and 12. The authors must clarify this point.

Referee #4

Nishimura and colleagues have made the new finding that Atg16L1, associated with the Atg12-5 complex, binds to FIP200, a member of the ULK kinase complex. This is an interesting and potentially important finding. Following a lead from a yeast 2 hybrid experiments they found Atg16 binds FIP200 weakly, such that the interaction of the endogenous proteins requires cross-linkers. Gel filtration of the cross-linked sample shows a large complex of Atg16L1 which is not present in FIP200 -/- MEFS. They go on to show that the FIP200 immunoprecipitated by Atg16L1 is associated with ULK1, and Atg13 suggesting the Atg16-interacting population of FIP200 is in the ULK complex. They next map the domain of Atg16L1 that binds FIP200 showing that it is the region in Atg16L1 between 230 and 300. They further show that deletion of this domain does not restore flux in the Atg16-/- MEFS. Conversely they show that this domain 230-300 is required for restoration of flux and proper co-localization on isolation membranes labelled with GFP-ULK1. Surprisingly, the Atg16L1 1- 230 construct, which does not bind FIP200, also restores flux but doesn't colocalize with GFP-ULK1 leading the authors to suggest it is deregulated and uses an alternative mechanism. Overall the paper is nicely written and the data is a very good quality, however the authors make a few speculations (such as that ULK complex and Atg12-5-16 complex maybe associated in cytosol, the 1-230 piece is deregulated) without really substantial proof. In addition, some of there data appears to contradict or ignore existing literature (p62 data from the authors lab, all the data on Rab33b published so far).
Major points:
1. Can the authors prove that FIP200 must be associated with the ULK complex to bind Atg16L1? This would appear to be essential for their model.
2. The authors should address the data from Fuduka concerning the Rab33b association with Atg16L1 as the domains to some extent overlap (Rab33b binds 141-265 of Atg16L1). Is Rab33b required for binding of the Atg16L1 to FIP200? If Rab33b is inactivated by OATL1 will the interaction be affected?
3. Similarly, does the binding of the 1-230 to membranes require Rab33b? The authors should ask if Atg16L1 1-230 colocalizes with GM130 as Rab33b is localized in part to the Golgi.
4. Fuduka made nice use of chimeras between Atg16L1 and L2 to show the functional difference.

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As the FIP200 binding site is absent in Atg16L2 can the authors confer FIP200 on Atg16L2 by making the appropriate chimera (adding 230-300 to Atg16L2)?
5. It is a bit puzzling that the levels of the ULK complex decrease with loss of Atg16L1 and this makes the conclusions complicated. Do the Atg16L1-/- MEFS have reduced levels of the ULK complex? In Fig. 4 using the Atg16L1-/- MEFS they observe that after the expression of the Atg16 1-230 GFP-ULK1 doesn't go to spots. Is this because the level of the other members (Atg13 and FIP200) are decreased? It would be informative to probe the levels of all members of the ULK complex in the cells used for Figure 4 panels A, B, and C as well as Fig, 5 as they all are slightly different MEFs, ie some are expressing pieces of Atg16, some pieces of Atg16 with GFP-ULK1 etc. This would pinpoint the role of the Atg12-5-16 complex rather than effects d=from destabilizing the ULK complex.
6. Do the authors have any idea why the ULK complex members levels decrease after loss of Atg16L1?

1st Revision - authors' response 29 December 2012

Responses to Reviewer #2

Characterization of the early events of autophagosome biogenesis is of great importance. Identification of an interaction between Atg 16 and FIP200 is very interesting, however the data presented in this study are too preliminary to support the authors' conclusions. Most importantly, the authors fail to convincingly demonstrate an effect of the different Atg16 mutants on overall autophagic activity.

<Response>

We would like to thank this reviewer for raising the valuable comments/criticisms. According to this general suggestion and the specific comments below, we have extended our studies and added many new data in this revised manuscript. In particular, we assessed autophagic activity of different Atg16L1 mutants using an additional method based on flow cytometry (please see our detailed reply to comment #5 below). We believe that these new data strengthen our conclusions.

Specific comments:

1. Figure 1D, the input amounts of FIP200, Atg13 and Ulk1 are not equal in the different conditions. This may affect the authors' interpretation. In addition, their interaction with other complex subunits seems to decrease while the interaction with Atg16 increases following the DSP treatment. The authors need to address this issue.

The FIP200 band in figure 1F is too weak and unclear.

<Response>

We agree with this concern. The ULK1, Atg13 and FIP200 protein levels in the cell lysates were reduced following high-dose DSP treatment (old Figure 1D). Treatment with 2 mM DSP might reduce detergent solubility of the ULK1 complex, resulting in the reduction in the lysate samples. In this revised manuscript, we show the result performed in the presence of a middle dose of DSP (1 mM) with a shorter incubation time (30 min) (new Figure 1D), in which both Atg16L1–FIP200 interaction and ULK1–Atg13–FIP200–Atg101 complex formation are clearly observed. We apologize for showing the unclear band of FIP200 in previous Figure 1F. We have replaced the immunoblot of FIP200 with a better one (new Figure 1F).

The authors should relate to the fact that FIP200 was not equally precipitated in all samples shown in Figure 1G. In contrast to the authors' conclusion, the ratio of FIP200 to Atg16 is different in the F/F vs. Δ/Δ samples.

<Response>

We apologize for not fully explaining this point. Interestingly, we found that FIP200 expression was enhanced in Atg14-deficient MEFs (see the input fraction in new Figure 1G). Therefore, it is difficult to equally precipitate FIP200 from WT and Atg14-deficient cell lysates. Instead, we examined the effect of the PI3K inhibitor wortmannin on the Atg16L1–FIP200 interaction, and found that this interaction was not significantly affected by wortmannin treatment (Figure 1F).

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Collectively, these results suggest that PtdIns(3)P is not a major factor for the Atg16L1–FIP200 interaction.

2. The experiment presented in Figure 2 supports reduction in individual protein but not complex stability directly as concluded by the authors. It is necessary to show complex formation by IP in the experiment presented in figure S2. It is also necessary to verify whether the decrease in protein stability is caused on the protein or mRNA level. Quantifications and statistical information should be performed.
<Response>
We would like to thank this reviewer for this suggestion. As suggested, we performed IP experiment using cell lysates derived from WT and Atg5 KO MEFs, and observed that ULK1 and Atg13 were co-immmuprecipitataed with FIP200 both in the absence and presence of Atg5. These results support our conclusion that formation of the ULK1–Atg13–FIP200 complex is not significantly affected in Atg5 KO MEFs. We have added the data to new Supplementary Figure S3B (previous Figure S2).

To verify whether the reduction of the ULK1 complex components in Atg5 KO cells is due to their protein destabilization or reduced mRNA levels, we examined degradation of the ULK1 complex components at different times following cycloheximide treatment. Reduction in protein levels of the ULK1 complex components was accelerated in the absence of Atg5 (Dox-treated condition) (new Figure 2C). In contrast, qPCR analyses showed that mRNA levels of the ULK1 complex components were not reduced in the absence of Atg5 (new Figure 2D). These results reinforce our conclusion that deficiency of Atg5 destabilizes ULK1, Atg13, and FIP200 proteins.

3. The authors should relate to the fact that in figure 3B FLAG-tagged deletions and HA-FIP200 levels are not equal. This makes it difficult to conclude that there is a decrease in their interaction with FIP200.
In addition, the authors need to relate/describe all the results shown in figures 3B and C.
<Response>
We apologize if the unevenness of tagged protein expression makes it difficult to understand our conclusion. Although the same dose of expression vectors were used for transfection and the same dose of cell lysates were used for immunoblot analysis, expression levels of the FLAG–Atg16L1 deletion mutants and HA-FIP200 were not equal. However, we did not intend to discuss this immunoprecipitation data quantitatively. We would like to show the region which is indispensable for the interaction with FIP200.The most important observation is that the deletion mutants lacking the 230-300 region did not co-immunoprecipitate FIP200 (Figure 3B), which cannot be explained by the expression variety of the tagged proteins. In addition, we showed another IP data in supplementary Figure S4B to narrow down the interaction domain, and found that 230-250 and 288-300 regions are important. Collectively, these data consistently suggest that the 230-300 region in Atg16L1 is essential for its interaction with FIP200. According to the reviewer's suggestion, we have added more detailed explanation of Figure 3B and 3C in the text.

4. The authors do not relate to the WB describing p62 in the different samples in figure 4A. For example, it is not clear why p62 levels decrease in the presence of chloroquine.
<Response>
We agree with this concern. As pointed out by the reviewer, chloroquine (20 μM) treatment did not inhibit p62 degradation in response to starvation (old Figure 4A). This could be because the lysosome-inhibitory effect of chloroquine is mild. p62 may be more sensitive to lysosomal degradation compared to LC3-II, and the remaining lysosomal activity could be sufficient to degrade p62. In line with this, recent paper reported that low dose chloroquine (10-30 μM) treatment is not enough for complete suppression of autophagic activity in HeLa cells (Ni et al., Vol. 7, 188-204, *Autophagy*, 2011). To address this issue, we used another lysosomal inhibitor bafilomycin A_1 ($BafA_1$), which is a more potent inhibitor than chloroquine. $BafA_1$ inhibited degradation of both LC3-II and p62 in response to starvation (new Figure 4A). We have replaced the previous immunoblotting data with the new one using $BafA_1$.

The authors did not specify the exact conditions for the experiment described in figure 4B.
<Response>
We apologize that we did not explain the experiment conditions. We now clearly state them in the legend.

The authors need to explain why they chose to use of GFP-ULK1 in figures 4B and 5 instead of FIP200, the subject of the current study.
<Response>
It is because GFP-ULK1 is most commonly used to detect localization of the ULK1–Atg13–FIP200–Atg101 complex, and therefore a best marker for the autophagosome formation site (Itakura et al., Vol. 6, 764-776, *Autophagy,* 2010; Kageyama et al., Vol. 22, 2290-2300, *Mol. Biol. Cell,* 2011; Itakura et al., Vol. 125, 1488-1499, *J. Cell Sci.,* 2012; Orsi et al., Vol. 23, 1860-1873, *Mol. Biol. Cell,* 2012). In addition, since GFP-ULK1 dots are not observed in FIP200 KO MEFs (Hara et al., Vol. 181, 497-510, *J. Cell Biol*., 2008), GFP-ULK1 should be included in the ULK1-Atg13-FIP200-Atg101 complex as well as endogenous ULK1 and FIP200. By contrast, less information is available for the use of GFP-FIP200 as a marker. That is why we chose GFP-ULK1 instead of FIP200 in these experiments.

5. To support the model proposed by the authors it is necessary to determine the different Atg16 mutants and FIP200 KO effect on autophagy. This should be performed by different assays including for example long-lived protein degradation.
<Response>
We would like to thank this reviewer for the valuable suggestion. To provide a stronger evidence for the effects of the different Atg16L1 mutants on overall autophagic activity, we performed a different assay using flow cytometry. It was reported that the total expression level of GFP-LC3 reversely correlates with autophagic degradation activity (Shvets et al., Vol. 4, 621-628, *Autophagy,* 2008). In Atg16L1 KO MEFs stably expressing GFP-LC3 and either full length Atg16L1(1-588) or Atg16L1(1-230), GFP fluorescence intensity was reduced following long-term starvation (6 h). The fluorescence level was completely restored by addition of wortmannin or bafilomycin A_1, confirming that the reduction in the GFP-LC3 signal depends on autophagy. By contrast, such a clear reduction was not observed in Atg16L1 KO MEFs expressing Atg16L1Δ (230-300). These results support our conclusion that Atg16L1(1-230), but not Atg16L1Δ(230-300), restores the autophagic defects of Atg16L1 KO MEFs. We have added these data together with quantification in new Figure 4 D and E. As described above (response to comment #4), the p62 data also suggest that Atg16L1Δ(230-300) is defective in induce autophagy (new Figure 4A).

<u>**Responses to Reviewer #3**</u>
The authors have done an excellent job. The manuscript convincingly demonstrates that FIP200 regulates several events during the autophagosome formation.
Minor point:
Figure 4 panel A. It is odd to observe that p62 does not accumulate in the presence of chloroquine in lanes 3,9 and 12. The authors must clarify this point.
<Response>
We would like to thank this reviewer for raising the valuable comment. This is the same comment as comment #4 of referee #2.

We agree with this concern. As pointed out by the reviewer, chloroquine (20 μM) treatment did not inhibit p62 degradation in response to starvation (old Figure 4A). This could be because the lysosome-inhibitory effect of chloroquine is mild. p62 may be more sensitive to lysosomal degradation compared to LC3-II, and the remaining lysosomal activity could be sufficient to degrade p62. In line with this, recent paper reported that low dose chloroquine (10-30 μM) treatment is not enough for complete suppression of autophagic activity in HeLa cells (Ni et al., Vol. 7, 188-204, *Autophagy*, 2011). To address this issue, we used another lysosomal inhibitor bafilomycin A_1 ($BafA_1$), which is a more potent inhibitor than chloroquine. $BafA_1$ inhibited degradation of both LC3-II and p62 in response to starvation (new Figure 4A). We have replaced the previous immunoblotting data with the new one using $BafA_1$.

Responses to Reviewer #4

Overall the paper is nicely written and the data is a very good quality, however the authors make a few speculations (such as that ULK complex and Atg12-5-16 complex maybe associated in cytosol, the 1-230 piece is deregulated) without really substantial proof. In addition, some of their data appears to contradict or ignore existing literature (p62 data from the authors lab, all the data on Rab33b published so far).

Major points:

1. Can the authors prove that FIP200 must be associated with the ULK complex to bind Atg16L1? This would appear to be essential for their model.

<Response>

We apologize that our IP and gel filtration analyses made the interpretation confusing. Considering that we identified FIP200 as an Atg16L1-interacting protein in two-hybrid analysis, we believe that FIP200 directly interacts with Atg16L1. To examine the requirement of ULK for the FIP200–Atg16L1 interaction, we performed an immunoprecipitation experiment using ULK1/2 double-knockout (DKO) MEFs and observed comparable levels of Atg16L1–FIP200 interaction in both WT and ULK1/2 DKO MEFs. This result suggests that association with the ULK1 complex is not required for the FIP200–Atg16L1 interaction. We have included this data in new supplementary Figure S2 and describe in the first paragraph of Result. Nonetheless, because FIP200 is mostly included in the ULK complex, it is reasonable to assume that Atg12—Atg5–Atg16L1 interacts with the whole ULK1 complex through FIP200 in the cell.

2. The authors should address the data from Fuduka concerning the Rab33b association with Atg16L1 as the domains to some extent overlap (Rab33b binds 141-265 of Atg16L1). Is Rab33b required for binding of the Atg16L1 to FIP200? If Rab33b is inactivated by OATL1 will the interaction be affected?

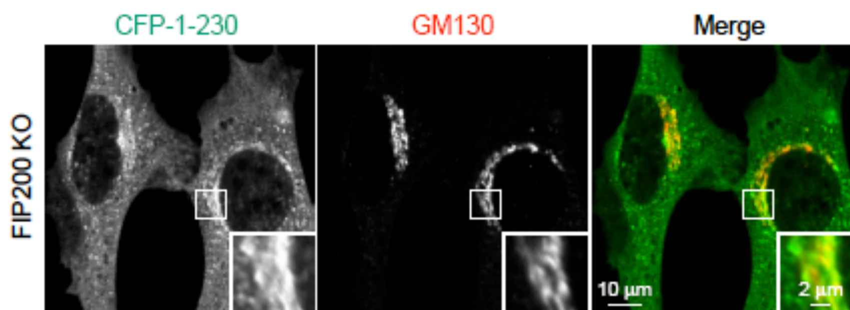
<Response>

We would like to thank this reviewer for the valuable comments. As suggested, we performed immunoprecipitation experiments using HEK293T cells stably expressing FLAG Rab33B, FLAG-Rab33B Q92L (a dominant active mutant), T7-OATL1 or T7-OATL1 R279K (a catalytic domain mutant). FIP200 co-immunoprecipitated Atg16L1, but not either FLAG-Rab33B or its Q92L mutant, indicating that Rab33B is not included in the Atg16L1–FIP200 complex. We also found that Rab33B inactivation by OATL1 overexpression did not significantly affect the Atg16L1–FIP200 interaction. Collectively, these results suggest that the Atg16L1–FIP200 interaction does not depend on Rab33B. We have added these data in Supplementary Figure S5.

3. Similarly, does the binding of the 1-230 to membranes require Rab33b? The authors should ask if Atg16L1 1-230 colocalizes with GM130 as Rab33b is localized in part to the Golgi.

<Response>

As shown in the below figure, a partial co-localization of Atg16L1(1-230) with GM130 was observed, indicating that this mutant partially localizes to the Golgi. However, because this is rather out of scope of this paper and there is no space remaining, we apologize that we cannot include these data in the revised version.



4. Fuduka made nice use of chimeras between Atg16L1 and L2 to show the functional difference. As the FIP200 binding site is absent in Atg16L2 can the authors confer FIP200 on Atg16L2 by making the appropriate chimera (adding 230-300 to Atg16L2)?

<Response>

The reviewer touches upon an interesting point that is the nature of physiological differences between Atg16L1 and Atg16L2. Although Atg16L2 can form a homo-oligomer and the Atg12—

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Atg5–Atg16L2 complex as well as Atg16L1, Atg16L2 does not localizes to isolation membrane and is dispensable for canonical autophagy (Ishibashi et al., 2011). These differences might be caused by different interaction partners including Rab33B and FIP200 (and perhaps additional factors). We think that this is a question that will constitute a study by itself. In future, we would like to perform more complete analysis on the difference between Atg16L1 and Atg16L2 with regards to their interacting partners.

5. It is a bit puzzling that the levels of the ULK complex decrease with loss of Atg16L1 and this makes the conclusions complicated. Do the Atg16L1-/- MEFS have reduced levels of the ULK complex?
<Response>
Yes, the expression levels of ULK1, Atg13, and FIP200 were reduced in Atg16L1 KO cells (Figure 2A).

In Fig. 4 using the Atg16L1-/- MEFS they observe that after the expression of the Atg16 1-230 GFP-ULK1 doesn't go to spots. Is this because the level of the other members (Atg13 and FIP200) are decreased? It would be informative to probe the levels of all members of the ULK complex in the cells used for Figure 4 panels A, B, and C as well as Fig, 5 as they all are slightly different MEFs, ie some are expressing pieces of Atg16, some pieces of Atg16 with GFP-ULK1 etc. This would pinpoint the role of the Atg12-5-16 complex rather than effects d=from destabilizing the ULK complex.
<Response>
We would like to thank this reviewer for this valuable comment. As pointed out by the reviewer, several different MEFs were used in these analyses. Therefore, it is difficult to distinguish whether expression levels of each ULK1 components are due to Atg16L1 deficiency or clonal variation among these cell lines. To address this issue, we examined the effect of wortmannin on ULK1 puncta formation, because wortmannin treatment induces accumulation of ULK1 puncta at the autophagosome formation site (Itakura et alk .,2010). ULK1 puncta formation was recovered in wortmannin-treated cells (Supplementary Figure S7), suggesting that the reduction of ULK1 puncta in Atg16L1 mutant cells is due to enhanced dissociation of ULK1 complex, but not due to a decrease in other members of the complex. However, as we cannot completely rule out a possibility that reduction in expression of ULK1 and other complex members partially contributes to the defect in Atg16L1 puncta formation, we state that both possibilities in the second last paragraph in Result.

6. Do the authors have any idea why the ULK complex members levels decrease after loss of Atg16L1?
<Response>
This is essentially the same question raised by Reviewer #2 (comment #2). To verify whether the reduction of the ULK1 complex components in Atg5 KO cells is due to their protein destabilization or reduced mRNA levels, we examined degradation of the ULK1 complex components at different times following cycloheximide treatment (we used Atg5 KO cells instead of Atg16L1 KO cells, but we believe that the mechanism should be the same). Reduction in protein levels of the ULK1 complex components was accelerated in the absence of Atg5 (Dox-treated condition) (new Figure 2C). In contrast, qPCR analyses showed that mRNA levels of the ULK1 complex components were not reduced in the absence of Atg5 (new Figure 2D). These results reinforce our conclusion that deficiency of Atg5 destabilizes ULK1, Atg13, and FIP200 proteins.

2nd Editorial Decision 16 January 2013

Thank you very much for sending us a revised version of your manuscript. As you will see from the referee comments pasted below, the referees agree that their concerns have been fully addressed. I am therefore very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

I would like to add that we would prefer to slightly modify the title for clarity to:

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"FIP200 regulates targeting of Atg16L1 to the isolation membrane". Please let us know if you have any objections about this. We would also like to move the information of the Statistical Analysis (currently in the Supplementary Information) to Materials and Methods in the main manuscript. We can do both these things ourselves, but please let us know if this is a problem.

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REFEREE REPORTS:

Referee #2
In the revised manuscript the authors addressed my remarks and added many important controls and experiments. In its present form the manuscript meets the scientific merit of EMBO Reports.

Referee #4
The authors have addressed all my concerns.

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