

Manuscript EMBOR-2014-39464

Starvation-induced MTMR13 and RAB21 activity regulates VAMP8 to promote autophagosome-lysosome fusion

Steve Jean, Sarah Cox, Sonya Nassari and Amy A. Kiger

Corresponding author: Amy A. Kiger, University of California, San Diego

Review timeline:

Submission date:	18 August 2014
Editorial Decision:	16 September 2014
Revision received:	10 December 2014
Accepted:	02 January 2015

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.)

Editor: Barbara Pauly

1st Editorial Decision

16 September 2014

Thank you very much for the submission of your research manuscript to our editorial office and for your patience while we were waiting to hear back from the referees. We have now received the full set of reviews on your manuscript.

As the detailed reports are pasted below I will only repeat the main points here. Referee 1 points out that it needs to be excluded that the observed effects are due to an alkalization of the lysosomes. This reviewer also states that Vamp8 trafficking to early and late endosomes should be analyzed at similar time scales. Reviewer 2 recommends testing whether Sbf can bind to Vamp7 and whether MTMR13/Sbf is necessary to connect Rab21, Vamp7 and Vamp8. Referee 3 feels that the effects of the proposed MTMR13/Rab21/Vamp8 axis on starvation-induced autophagy should be analyzed in more detail. All reviewers point out some instances in which additional controls and clarifications are needed. Finally, while it seems beyond the scope of the current paper to test whether MTMR13 can act as a GEF for all Rab's (referee 2), it might be worth analyzing whether it does so for Rab28.

Overall, and given the reviewers' constructive comments, I would like to give you the opportunity to

revise your manuscript, with the understanding that the main concerns of the reviewers should be addressed, especially the point about the alkalization of the lysosomes raised by referee 1. 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. If you feel that this period is insufficient for a successful submission of your revised manuscript I can potentially extend this period slightly.

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 #1:

In their manuscript, Jean et al study the regulation of the autophagosome-lysosome membrane fusion process. The authors provide evidence that upon starvation activated Rab21 directly binds Vamp8, a SNARE protein known to be involved in the fusion between the autophagosomal and the lysosomal membranes. They further found that upon starvation Rab21 is activated by MTMR13, a GEF of Rab21. Accordingly, the authors propose that starvation promotes the upregulation of Vamp8 trafficking to the lysosome where it is essential for the fusion of autophagosome with lysosome.

Characterization of the SNARE complexes involved a late stage of autophagy, namely production of autolysosome vesicles is both important and of wide interest. The authors describe for the first time the involvement of the small GTPase Rab21 and its nucleotide exchange factor, MTMR13 in this step. However, to demonstrate a direct link between these factors and autophagy, it is important to exclude the possibility that the entire phenomenon described in this study is a consequence of the inability of lysosomes to remain acidic and active under these conditions. This is particularly important in light of the data presented in figure 1, which imply that lysosomal acidification was indeed affected by the different treatments. Of note, the acidity of lysosomes is known to differ from that of autophagosomes, which should be taken under consideration while using LysoTracker.

Additional remarks:

Figure S2- the authors follow the distribution of markers of subcellular compartments upon Rab21 and Sbf knockdown. Unlike the description in the text, the siRNA of the different proteins shows a differential effect on the subcellular markers. While knocking down Sbf shows a strong effect on Rab7 (S2G), Lamp1 is affected by Rab21 siRNA. These differences should be taken under consideration.

Figure 3- the authors compare the accumulation of Vamp8 to early endosomes (panel 3N) to that of late endosomes (panel 3Q) yet the time scales are different. It would be informative to compare the trafficking of both upon equal time scales. In this regard, it should also be shown that the colocalization of Vamp8 with Rab7 is affected upon Rab21 and MTMR13 siRNA.

The authors show in figure S2H-K that Rab21 and Sbf siRNA affect the distribution of Lamp1. This might cause an indirect effect on its colocalization with Vamp8 in figure 3S-V.

Minor comments-

- Figure S2A-C- an autophagic marker should be used to determine the effect of Vamp7 siRNA on autophagy and its localization on autophagosomes.
- Figure S2Z- the authors suggest a lysosomal degradation of Vamp8, which should be demonstrated

by western blot.

- The figures presented are very large and compact. It would be beneficial to the reader if the authors would separate them into smaller figures.

Referee #2:

In this article, the authors characterized the function of Sbf/MTMR13 and Rab21 in autophagy in drosophila and HeLa cells. They show that Sbf/MTMR13 and Rab21 have a conserved function for autophagy, regulate the traffic of VAMP7 in the fly and VAMP8 in HeLa cells, and further found regulation of these molecular networks by starvation. The results presented here are novel and of high interest. The experiments were well carried out and controlled. Nevertheless I see major questions which need to be addressed in order to get a clear-cut message.

1-Sbf/MTMR13 is presented as a Rab21 GEF but others found it as a GEF of also Rab28, which was involved in the degradative pathway. At this point, it is required to test Sbf/MTMR13 GEF activity against all Rabs. Several groups have the whole Rab mutant collection available so this is likely not so difficult to achieve by testing binding to CA vs DN mutants of Rabs to Sbf/MTMR13.

2-the fly data indicate a clear connection with Vamp7 but the authors did not assay for binding of Sbf to Vamp7: could Sbf be the fly Varp?

3-along the same line, the interaction between Rab21 and Vamp7 in mammalian cells presumably requires Varp. From the authors' data it is not clear whether or not Sbf/MTMR13 is a required molecular link between Rab21 and Vamp7 and Vamp8. This should be tested preferably with recombinant fragments or in vitro translated proteins so as to get a hint of what are the direct interactions.

4-in mammalian cells, longer times of starvation (like for Vamp8, figure 4A) should be tested in the case of Vamp7. The authors may also test other inducers of autophagy to ascertain their conclusion regarding the involvement of Vamp8 not Vamp7.

The author should change their title and abstract to include the notion that it is Vamp7 which is involved in fly. As it is the message conveyed by the title and abstract is misleading.

Referee #3:

In this manuscript, the authors expanded their previous findings (Mol Biol Cell, 14: 2723-2740, 2012) and found that activation of RAB21 GTPase through Sbf/MTMR13 GEF (guanine nucleotide exchange factor) and subsequent endosomal trafficking of VAMP8 SNARE (soluble NSF attachment protein receptor) are involved in autophagy at fusion step of autophagosomes with lysosomes. They further showed increased interactions of Sbf/MTMR13 with RAB21 and RAB21 with VAMP8 on late endosome in response to starvation. Based on these observations, the authors claimed that the endolysosomal sorting machinery upon nutrient-deprivation contributes to the regulation of autophagy. This is an interesting study, well performed and the manuscript is well written. Novel aspects are, as mentioned above, specification of a novel GEF-Rab-effector pathway. Major comments

1. The results shown in Figs. 1 and 2 suggest that Sbf/MTMR13 and Rab21 are required for not only starvation-induced but also basal/constitutive autophagy. Nevertheless, the authors emphasized a role of MTMR13-RAB21 pathway in starvation-induced autophagy. This pathway is not involved in basal autophagy? In addition, they showed that the starvation increases the interaction of Sbf/MTMR13 with RAB21 and/or RAB21 with VAMP8, but the consequence was not provided. The authors should demonstrate the correlation between autophagy and dynamics of VAMP8 in response to starvation by showing the autophagic activity with LC3-II and p62 degradation, and the cellular localization of VAMP8.

2. In Fig. 4, the authors showed that RAB21-GTP has high affinity for VAMP8. While the GTP form of RAB21 was gone after starvation for 60min, the interaction of RAB21 with VAMP8 was observed even at 90min after starvation. The authors should explain this discrepancy. According to

the authors' model, the active form of RAB21 (RAB21CA) should bind to VAMP8 constitutively, but such interaction is apparently enhanced by the starvation as shown in Fig. 4B. The authors

should describe this reason.

3. In Fig. S4, the interaction of RAB21 with VAMP8 was induced under both serum- and glucose-deprived conditions but not sole deprivation, implying the complicated regulation of fusion machinery between autophagosomes and lysosomes dependent on extracellular nutrient conditions.
4. In Fig. 2N-Q, the autophagy-flux for 2 hours was estimated, but the time is too short to judge the autophagy-flux under nutrient-rich conditions. The authors should perform the autophagy-flux assay with longer time.
5. Fig. 3G lacks control immunoblots for MTMR13 and RAB21 ensuring RAB21KD and MTMR13KD, respectively.
6. In Fig. 3 C and D, the authors should show each image for Lysotracker and GFP-VAMP7 in addition to the merged color image.

Minor comments

1. In Fig. 1, the authors' claim would be consolidated by double-immunofluorescence analysis for LC3 and Ref(2)P.
2. In Fig. 1I, the authors should show immunoblot data for LC3.
3. In Fig. 1J, is there statistically significant difference?
4. Page 7, line 3: Figure numbers should be replaced (IH with 1H, IJ with 1J).
5. Page 7, line 5 from the bottom: "the mutant" should be substituted with "the knock-down".
6. Page 8, line 2: the authors should discuss about not only increased number but also the size of autophagosome.
7. Page 8, subheading "Conserved human roles~" is not clear.

We would like to thank all Referees for their time and feedback, which has led to a clearer and stronger manuscript.

Responses to Referee Comments

Referee #1

1. **“...It is important to exclude the possibility that the entire phenomenon described in this study is a consequence of the inability of lysosomes to remain acidic and active under these conditions. This is particularly important in light of the data presented in figure 1, which imply that lysosomal acidification was indeed affected by the different treatments.”**

We have addressed this concern about the possibility of a primary role in lysosomal acidification through several experiments. First, we now include data showing that there are similar numbers of LysoTracker-marked acidified compartments in *Rab21* or *Sbf/MTMR13* knockdown conditions as in control cells, as detected in both fed HeLa cells and fly hemocytes (macrophages) (**Figure 3A-3F; text p. 9**, under new section, “*Rab21* and *Sbf/MTMR13* are not required for general lysosome biogenesis or function”). If anything, slightly more LysoTracker objects were present in the knockdown versus control conditions (**Figure E2R**). Second, we also measured the extent of acidification using LysoSensor probe in starved HeLa cells. This indicated a similar average vesicular pH in starved control and siRNA cells (**Figure 3G-3J; text p. 9**). Moreover, the LysoTracker and LysoSensor results for *RAB21* or *MTMR13* knockdown were comparable to those seen with *VAMP8* siRNA (**Figure 3J, Figure E2R**). In addition, we showed that the rates and levels of EGF-induced EGFR turnover that depends on normal lysosomal acidification and degradative function (Ganley et al, 2011) still occurs normally in *RAB21* and *MTMR13* knockdown cells (**Figure 3O-3R**).

We would like to note (as mentioned in the text, **pages 5, 6 and 8**) that *unlike* most human and fly cells, the wildtype fly fat body cells exhibit an unusual lack of acidified lysosomes in fed larvae. Larval starvation normally induces autophagy and acidified autolysosomes (Rusten et al, 2004; Scott et al, 2004), which were blocked by *Sbf* or *Rab21* knockdown (**Figure 1A-1D**; and similarly observed in many other autophagy mutants (Juhász et al, 2008; Rusten et al, 2004; Scott et al, 2004; Takats et al, 2013)). In contrast to the fly fat body, we observed normal lysosomal acidification in fly hemocytes and human cells depleted of *Rab21* or *Sbf/MTMR13* (above). Together, these results indicate that *Rab21* and *Sbf/MTMR13* do not play a conserved role required for lysosomal acidification *per se*, including in the context of human cell autophagy.

2. **“Figure E2- the authors follow the distribution of markers of subcellular compartments upon *Rab21* and *Sbf* knockdown. Unlike the description in the text, the siRNA of the different proteins shows a differential effect on the subcellular markers. While knocking down *Sbf* shows a strong effect on *Rab7* (E2G), *Lamp1* is affected by *Rab21* siRNA. These differences should be taken under consideration.**

Although not identical, the *Rab21* and *Sbf* RNAi phenotypes in flies were qualitatively similar to one another with statistically significant effects on *Rab7* and LAMP1 (**Figure E3E-E3L**). In contrast, LAMP1 and LAMP2 compartment number, size and distribution (**Figure 3K-3N, Figure 4R-4T**) and general endolysosomal trafficking (**Figure 3O-3R, EGFR**) appeared to be unaffected in *RAB21*- or *MTMR13*-depleted HeLa cells. This indicates that general effects on endolysosomal compartments are unlikely to be the basis of conserved starvation-regulated

requirements for *Sbf/MTMR13* and *Rab21* in autophagy. In contrast, shared defects in Vamp7/VAMP8 distribution in *Sbf/MTMR13* and *Rab21* knockdowns highlight their conserved roles specifically in SNARE trafficking from flies to humans (**Figure 4A-4D, 4M-4U**).

We previously showed that Sbf has roles in both PI(3)P regulation and Rab21 activation (Jean et al, 2012). Thus, Sbf contributions to PI(3)P-regulated trafficking independent of Rab21 could account for some phenotypic differences. In fact, we found opposite effects of accumulated versus depleted PI(3)P levels in cells with *Sbf* or *Rab21* RNAi, respectively ((Jean et al, 2012) and not shown), indicating PI(3)P regulation is unlikely to explain the shared Sbf and Rab21 roles in autophagosome-lysosome fusion. We now briefly discuss the possibility of Sbf PI(3)P-mediated phenotypes independent of Rab21 (**text p. 16**).

3. “Figure 3- the authors compare the accumulation of Vamp8 to early endosomes (panel 3N) to that of late endosomes (panel 3Q) yet the time scales are different. It would be informative to compare the trafficking of both upon equal time scales.”

To address this question, we now show VAMP8 distribution at early and late endosomes under the same starvation timecourse (0, 30 and 60 min starvation; now **Figure 4E-4L**). These results more clearly substantiate that, although there is no detectable difference in VAMP8 (or RAB21) co-localization with EEA1 under 30-60 minutes starvation (**Figure 4E-4I**), there is a small but significant increase in VAMP8 colocalization with RAB7 (**Figure 4I-4L**). Similar results in the starvation response of VAMP8 localization at early endosomes (unaffected) or late endosomes (enhanced) were also detected by independent methods (**Figure E3Dd**).

4. “...In this regard, it should also be shown that the colocalization of Vamp8 with Rab7 is affected upon Rab21 and MTMR13 siRNA.”

This is an interesting question, and we have now analyzed VAMP8 late endosomal localization in knockdown conditions. With either *RAB21* or *MTMR13* siRNA depletion, we found that the degree of VAMP8 and RAB7 colocalization dropped to nearly half of that seen in starved control cells (**Figure 4M-4P; text p. 11**). This is consistent with a starvation-induced role for Rab21 and MTMR13 functions to promote the starvation-induced VAMP8 accumulation at late endosomes.

5. “The authors show in figure E2H-K that Rab21 and Sbf siRNA affect the distribution of Lamp1. This might cause an indirect effect on its colocalization with Vamp8 in figure 3S-V.”

The different data mentioned above came from flies (**Figure E3I-E3L**, showing Lamp1 distribution) and humans (**Figure 4R-4U**, showing VAMP8 uptake and trafficking to LAMP1). Unlike the Lamp1 data referred to in flies, both LAMP1 and LAMP2 distribution appeared to be unaffected in human cells with *RAB21* or *MTMR13* depletion (**Figure 4R-4T, Figure 3K-3N**). Thus, a conserved basis for their roles in autophagosome-lysosome fusion did not appear to be due to general effects on a lysosomal biogenesis or general functions e.g., acidification, EGFR degradation (**Figure 3D-3R**). Our data do support a specific role in human cells for an MTMR13-RAB21 pathway in VAMP8 (but not VAMP7) regulation.

In flies, both Lamp1 and Vamp7 distribution along with lysosomal maturation appear to be affected with *Rab21* or *Sbf* knockdown. This could be related to possible broader roles for the fly Sbf GEF, Rab21 GTPase and/or Vamp7 that are fulfilled in humans by multiple distinct members of these protein families, such as a specific role for the mammalian VAMP7 homolog

in endosomal-lysosomal fusion (Pryor et al, 2004). Due to the single fly Vamp7 representing both human VAMP7 and VAMP8 homologs, we agree that the primary versus secondary consequences are more difficult to tease out in flies alone. This is why we migrated our questions and assays related to regulation of VAMP8 trafficking to human cells. Importantly, both flies and human cells show a starvation-induced Sbf/MTMR13 GEF and Rab21 GTPase activation, along with shared defects in autophagosome clearance.

Minor comments:

6. “Figure E2A-C- an autophagic marker should be used to determine the effect of Vamp7 siRNA on autophagy and its localization on autophagosomes.”

Roles for Vamp7 in autophagosome-lysosome fusion in the *Drosophila* fat body was recently published by others (Takats et al, 2013), with similar results to the role shown for VAMP8 in mammalian cells (Furuta et al, 2010; Itakura et al, 2012). Neither human VAMP7 nor VAMP8 normally localize to autophagosomes (**Figure E4G-E4I**, (Itakura et al, 2012)).

7. “Figure E2Z- the authors suggest a lysosomal degradation of Vamp8, which should be demonstrated by western blot.”

The data referred to above show a decrease in colocalization between VAMP8 and LAMP1 in response to starvation, which we speculated might be due to the increased autophagy flux. We did not mean to imply by ‘turnover’ that VAMP8 itself was degraded in the lysosome, but rather that the colocalization was more dynamic. To clarify our intent, we have revised the word ‘turnover’ to ‘dynamics’ (**text p. 11**).

8. “The figures presented are very large and compact. It would be beneficial to the reader if the authors would separate them into smaller figures.”

In response to this Reviewer’s comment, we have reorganized the data into six Figures and five Supplemental Figures. If necessary upon Reviewer and Editor advisement, we would consider removing some data panels, such as the low magnification image panels of VAMP8 and RAB7 (**Figure 4M, 4N, 4O**) and VAMP8 and LAMP1 (**Figure 4R, 4S and 4T**).

Referee #2

1. “Sbf/MTMR13 is presented as a Rab21 GEF but others found it as a GEF of also Rab28, which was involved in the degradative pathway. At this point, it is required to test Sbf/MTMR13 GEF activity against all Rabs. Several groups have the whole Rab mutant collection available so this is likely not so difficult to achieve by testing binding to CA vs DN mutants of Rabs to Sbf/MTMR13.”

Our work previously discovered that fly Sbf is a GEF for Rab21 (Jean et al, 2012). Here, we extend our findings to human MTMR13 and, importantly, delve into the shared function and mechanism for a conserved Sbf/MTMR13 and Rab21 pathway in autophagy. In this respect, our findings do not address nor depend on the previous report of human MTMR13 GEF activity for RAB28 (Yoshimura et al, 2010). However, an important relevant question to our current study raised from this other work is whether an MTMR13-RAB28 activity is significant to an MTMR13

function in autophagy?

To address the significance of RAB28 to the MTMR13-RAB21 autophagy functions, we performed a series of *RAB28* knockdown experiments with autophagy assays in HeLa cells (**Figure E2; text p. 8**). We found that, unlike with *MTMR13* and *RAB21* depletion, the *RAB28*-depleted cells exhibited normal LC3-II processing, both in untreated and bafilomycin-treated conditions (**Figure E2J-E2L**). Both control and *RAB28* siRNA cells accumulated LC3-II to similar levels in response to bafilomycin inhibition of autophagosome-lysosome fusion, suggesting that autophagosome formation and flux are independent of *RAB28* function. In a microscopy-based assay, we observed a reduction in the number of GFP:LC3 marked objects indicative of fewer autophagosomes in *RAB28* siRNA versus control cells (**Figure E2M, E2O, E2Q**). This is in contrast to the accumulation of LC3-II and number of GFP:LC3 objects seen with either *MTMR13* or *RAB21* siRNA (**Figure 2**). The lack of a shared requirement suggests that *MTMR13* *RAB28* GEF activity is unlikely to play a shared role in autophagosome-lysosome fusion.

Importantly, there is no clear *RAB28* homolog encoded in *Drosophila*. Our study focused on a conserved pathway utilizing Sbf/MTMR13 GEF activity in autophagy. Thus, *RAB28* is a poor candidate for illuminating the conserved Sbf/MTMR13 requirements for autophagosome-lysosome fusion in flies and humans.

Finally, testing Sbf/MTMR13 GEF activity against all Rabs is not a trivial undertaking and is beyond the scope of our study. The biochemical survey has been done, as noted, from which *RAB28* (but not *RAB21*) was identified (Yoshimura et al, 2010). It is becoming broadly appreciated that a single GEF can act independently on multiple distinct Rabs (Barr, 2013; Jean & Kiger, 2012), as well as that a single Rab may perform multiple functions under the regulation of multiple distinct GEFs (Mizuno-Yamasaki et al, 2012). It is interesting in future work to address what is the function for a *MTMR13* GEF activity for *RAB28*, and how the various *MTMR13* GEF functions are specifically regulated. Here, our work advances that *MTMR13* *RAB21* GEF activity is regulated by starvation and promotes *MTMR13*, *RAB21* and *VAMP8* interactions important for autophagy.

2. “The fly data indicate a clear connection with Vamp7 but the authors did not assay for binding of Sbf to Vamp7: could Sbf be the fly Varp?”

We have now tested binding between fly Sbf and fly Vamp7. Our results indicate that there is a striking starvation-induced Sbf and Vamp7 interaction in whole starved fly larvae (**Figure 5G; text p. 13**). In addition, we now show that there is also a starvation-induced Rab21 and Vamp7 interaction in whole starved larvae (**Figure 5F**). Both of these results are consistent with our model for a conserved Sbf/MTMR13 and Rab21 pathway that regulates *VAMP8/Vamp7* for an autophagy response to starvation. The starvation-induced interactions are analogous to those seen between *MTMR13*, *RAB21* and *VAMP8*, and are unlike the starvation insensitive interaction between *RAB21* and *VAMP7* (**Figure 5A**).

The Sbf protein is the closest homolog in flies to human *MTMR13/MTMR5* proteins, with homology throughout the length of the proteins (42/61% and 42/58% identity/similarity, respectively, (Jean et al, 2012)). By sequence alignments, there are no obvious amino acid features shared between Sbf and VARP. In addition, there is no obvious VARP homolog in *Drosophila* (by standard database and alignment methods). By functional comparison, we observed parallel roles for fly and human Sbf and *MTMR13* in starvation-induced Rab21 activation and *VAMP8/Vamp7* trafficking in autophagy. This suggests that the autophagy role for fly Sbf-Rab21 activity is most akin to a human *MTMR13-RAB21* rather than a VARP-RAB21 function. However, this does not rule out whether there is an unknown role for VARP in autophagy?

To more directly address the possible significance of VARP to a RAB21 activity required for autophagy, we tested the effects of *VARP* depletion in HeLa cells. We found that, unlike with *MTMR13* and *RAB21* depletion, the *VARP*-depleted cells exhibited normal LC3-II processing in both untreated and bafilomycin-treated conditions (**Figure E2H-E2I; text p. 8**). Both control and *VARP* siRNA cells accumulated LC3-II to similar levels in response to bafilomycin inhibition of autophagosome-lysosome fusion, suggesting that autophagosome formation and flux are independent of *VARP* function. In a microscopy-based assay, we observed a reduction in the number of GFP:LC3 marked objects indicative of fewer autophagosomes in *VARP* siRNA versus control cells (**Figure E2M, E2N, E2P**). This is opposite to the accumulation of LC3-II and number of GFP:LC3 objects seen with either *MTMR13* or *RAB21* siRNA (**Figure 2**). The lack of a shared requirement suggests that VARP GEF activity is unlikely to play a role in RAB21-dependent autophagosome-lysosome fusion, further supporting that Sbf is not acting as VARP for its autophagy role in flies. On the other hand, the observed *VARP*-dependent decrease in GFP:LC3 puncta is more reminiscent to the role for human *VAMP7* involved in autophagosome formation (Moreau et al, 2011), and thus suggestive of a VARP-VAMP7 role in the regulation of an early step in autophagy.

3. “Along the same line, the interaction between Rab21 and Vamp7 in mammalian cells presumably requires Varp. From the authors' data it is not clear whether or not Sbf/MTMR13 is a required molecular link between Rab21 and Vamp7 and Vamp8. This should be tested preferably with recombinant fragments or in vitro translated proteins so as to get a hint of what are the direct interactions.”

Whether there is direct binding between MTMR13 and VAMP8 is an interesting question for future studies.

Here, we clearly showed that there is a required molecular link between MTMR13, Rab21 and VAMP8 for autophagy: (1) Sbf/MTMR13 binds to Rab21 in response to starvation; (2) Both Sbf/MTMR13 and Rab21 bind VAMP8 in response to starvation; (3) Rab21 activity increases with starvation; (4) MTMR13 function is required for starvation-induced RAB21 activity, RAB21-VAMP8 interaction, and VAMP8 endolysosomal trafficking; and (5) Sbf/MTMR13, Rab21 and VAMP8 are each required for autophagosome-lysosome fusion. Although Rab21 binds both VAMP7 and VAMP8, we found that only the Rab21 interaction with VAMP8 is starvation-induced along with autophagy. As discussed above, the focus of our study is on the molecular and functional interactions between Sbf/MTMR13, Rab21 and VAMP8 with respect to their shared and interdependent roles in autophagy.

4. “In mammalian cells, longer times of starvation (like for Vamp8, figure 4A) should be tested in the case of Vamp7. The authors may also test other inducers of autophagy to ascertain their conclusion regarding the involvement of Vamp8 not Vamp7.”

We now show an equivalent starvation timecourse for both VAMP8 and VAMP7 binding to RAB21, which shows that the VAMP7-RAB21 interaction remains unenhanced by starvation throughout the 90 minute timecourse (**Figure 5A**). Previous studies indicated potential VAMP7 roles in early autophagosome formation (Itakura et al, 2012; Moreau et al, 2011; Puri et al, 2013), distinct from *MTMR13* and *RAB21* required for autophagosome-lysosome fusion that is the focus of this study.

5. “The author should change their title and abstract to include the notion that it is

Vamp7 which is involved in fly. As it is the message conveyed by the title and abstract is misleading.”

We agree that the nomenclature between flies and humans makes it confusing to follow. Fly Vamp7 is the single homolog of both VAMP7 and VAMP8, so it is possible that it fulfills roles performed independently by each in humans. In flies, Vamp7 was shown, like human VAMP8, to act in a SNARE complex with Syntaxin17 and SNAP29 for autophagosome-lysosome fusion (Takats et al, 2013). Our work addresses this specific VAMP8 autophagy role for fly Vamp7 in autophagy. In this respect, we believe it is even more misleading to have Vamp7 in the title. To maintain clarity, the title is specific for the human pathway. We have added a clarification about fly Vamp7 in the abstract (**text p. 2**), in addition to when Vamp7 is first introduced in the Results (**text p. 9**).

Referee #3

1. “The results shown in Figs. 1 and 2 suggest that Sbf/MTMR13 and Rab21 are required for not only starvation-induced but also basal/constitutive autophagy. Nevertheless, the authors emphasized a role of MTMR13-RAB21 pathway in starvation-induced autophagy. This pathway is not involved in basal autophagy?”

Yes, our data indicates that the pathway is involved in basal as well as starvation-induced autophagy, as shown by the accumulation of p62 in flies and LC3-II in HeLa cells under fed knockdown conditions (**Figures 1E-1J, Figure 2I-2Q**). We now clarify this in the text on **page 6** (“These results point to a requirement for *Rab21* and *Sbf* functions in both basal and starvation-induced autophagy.”), on **page 7** (“As in flies, specific *RAB21* or *MTMR13* siRNA depletion in HeLa cell basal conditions (Figure E2A-E2B) led to a similar abnormal increase in the number of autophagosomes indicated by GFP:LC3 (human Atg8; Figure 2I-2M”), and as previously mentioned on **page 14** (“Although *Rab21* and *Sbf/MTMR13* are required for VAMP8 endolysosomal trafficking and autophagy in both basal and starvation-induced conditions, the starvation conditions specifically enhanced regulatory protein interactions seen between *Sbf/MTMR13*, *Rab21* and VAMP8.”).

That the pathway is also starvation-induced is significant for understanding regulation of flux (**Figure 5-6**), so we believe that it is an important point that should be emphasized.

2. “In addition, they showed that the starvation increases the interaction of Sbf/MTMR13 with RAB21 and/or RAB21 with VAMP8, but the consequence was not provided.”

To address the consequences of starvation-enhanced *Sbf/MTMR13* and *RAB21* interaction, we tested *RAB21* activity directly. Using a pull-down assay for activated *RAB21*-GTP (that selectively binds APPL1 (Zhu et al, 2007)), we show that starvation increases *RAB21* activity that depends on *MTMR13* function (**Figure 6A-6C**). We showed consequences of starvation-enhanced *RAB21* and VAMP8 interaction through VAMP8 localization and trafficking assays. We found that *MTMR13* and *RAB21* are required for starvation-induced VAMP8 colocalization with *RAB7* late endosomes (**Figure 4M-4P**) and for VAMP8 endosomal trafficking to lysosomes (**Figure 4R-4U**). Finally, each is required for an autophagosome-lysosome fusion step in autophagy (**Figure 1-2, Figure E3A-E3C** and (Furuta et al, 2010; Itakura et al, 2012; Takats et al, 2013)).

3. “The authors should demonstrate the correlation between autophagy and dynamics of VAMP8 in response to starvation by showing the autophagic activity with LC3-II and p62 degradation, and the cellular localization of VAMP8.”

We are not sure that we understand the comment. We showed that VAMP8 shares little colocalization with Atg5 or LC3 in HeLa cells (**Figure E4G-E4I**). This perhaps is expected given VAMP8 endolysosomal localization. Given the low degree of colocalization between VAMP8 and LC3, we did not assess the triple colocalization between VAMP8, LC3 and p62.

4. “In Fig. 4, the authors showed that RAB21-GTP has high affinity for VAMP8. While the GTP form of RAB21 was gone after starvation for 60min, the interaction of RAB21 with VAMP8 was observed even at 90min after starvation. The authors should explain this discrepancy. According to the authors’ model, the active form of RAB21 (RAB21CA) should bind to VAMP8 constitutively, but such interaction is apparently enhanced by the starvation as shown in Fig. 4B. The authors should describe this reason.”

Importantly, both RAB21 activation and VAMP8 interaction showed an early response to starvation within the same timeframe (15-30 minutes; **Figure 5A, Figure 6A**). We believe that the kinetic differences following induction are due to procedural differences underlying the distinct experiments looking at RAB21-GTP activity or effector interactions. For example, the buffers used in the two experiments were different (**Methods**). Also, we and others have observed that Rab21-GTP has a relatively rapid rate of hydrolysis, so it is possible that increased expression levels of VAMP8 could stabilize Rab21-GTP in the co-immunoprecipitation experiment. We have now added to the text a comment on the observation that starvation enhances Rab21-CA and VAMP8 binding, which suggests that other factors may be required to stabilize this protein interaction (**text p. 13**: “These results suggest that starvation promotes RAB21 activity and enhanced protein association with VAMP8.”).

5. “In Fig. E4, the interaction of RAB21 with VAMP8 was induced under both serum- and glucose-deprived conditions but not sole deprivation, implying the complicated regulation of fusion machinery between autophagosomes and lysosomes dependent on extracellular nutrient conditions.”

Yes, there were low levels of interaction between RAB21-VAMP8 under all conditions (consistent with basal roles, above), but only the full starvation lacking both serum and glucose led to an enhanced interaction. A deeper understanding of the mechanisms that regulate starvation-induced Sbf/MTMR13 and RAB21 activity are of great interest for our future directions.

6. “In Fig. 2N-Q, the autophagy-flux for 2 hours was estimated, but the time is too short to judge the autophagy-flux under nutrient-rich conditions. The authors should perform the autophagy-flux assay with longer time.”

We repeated the autophagy flux experiments with a five hour timepoint (see below, Figure A). We did observe further enhanced levels of LC3-II in the untreated RAB21 or MTMR13 deficient conditions at five hours (Figure A) compared to two hours starvation (**Figure 2N-2Q**), consistent with an accumulation of autophagosomes in the knockdown versus control cells (white bars).

Similarly, we also see at the longer timepoint that there are further increased levels of LC3-II with bafilomycin treatment (with a ratio LC3-II/tubulin >5 at five hours versus >2 at two hours, respectively). Together, the different timepoints both demonstrate a similar accumulation of LC3-II levels in the control and siRNA depleted cells, further supporting that the defects occur at a step in autophagy flux. Importantly, VAMP8 siRNA shows a similar result at both timepoints (below). Due to space constraints and the same conclusions from both timepoints, we have opted to include only the two hour timepoint that provides the clearest visualization of the data.

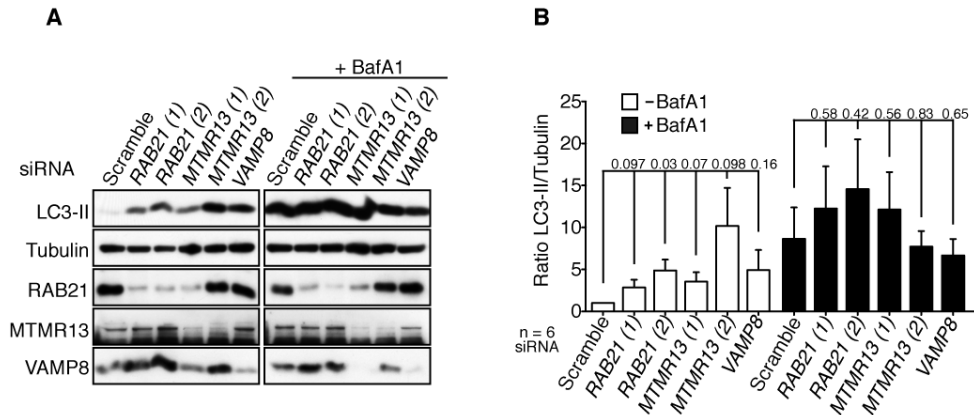


Figure A: Requirement for RAB21, MTMR13 and VAMP8 in autophagic flux. (A) RAB21, MTMR13 and VAMP8 are required for long-term LC3-II flux in HeLa cells starved for 5 hours. Anti-LC3 immunoblot of RAB21, MTMR13 or VAMP8 siRNA-depleted HeLa cells grown in full media with or without Bafilomycin A1. (B) Ratio of LC3-II to tubulin integrated densities from six independent experiments; SEM.

7. “Fig. 3G lacks control immunoblots for MTMR13 and RAB21 ensuring RAB21KD and MTMR13KD, respectively.”

There may have been a misunderstanding of the data shown. RAB21 and MTMR13 protein depletion with knockdown were shown on the third and fourth row, respectively, of the panel. For the sake of space, we had condensed the individual experiments into one panel. Now, we include all the data from within each RAB21 and MTMR13 knockdown experiments (Figure 3O, 3Q). The knockdown of RAB21 or MTMR13 did not appreciably impact the expression level of the other, respectively (Figure E2A-E2B).

8. “In Fig. 3 C and D, the authors should show each image for LysoTracker and GFP-VAMP7 in addition to the merged color image.”

We now include the data for LysoTracker and GFP:Vamp7 co-stain, which shows that LysoTracker remains absent in the Rab21 or Sbf knockdown conditions (Figure 4A-4D; text p. 10). However, we decided to show this only as merged data due to several reasons, including space constraints, the request from Reviewer 1 to make the Figures less dense and the similar LysoTracker results shown as individual channel images in Figure 1.

Minor comments

1. “In Fig. 1, the authors' claim would be consolidated by double-

immunofluorescence analysis for LC3 and Ref(2)P.”

We now include data showing Atg8 and Ref(2)P colocalization (**Figure E1G-E1I**). The results indicate that *Rab21* or *Sbf* knockdown leads to an accumulation of colocalized Atg8 and Ref(2)P, consistent with a block in autophagosome clearance.

2. “In Fig. 1I, the authors should show immunoblot data for LC3.”

We have shown that autophagosome flux detected by LC3-II levels on immunoblots is blocked in human cells with *MTMR13* or *RAB21* depletion (**Figure 2N-2Q**). We also demonstrated that autophagosome flux as detected by mCherry:GFP:Atg8, a fluorescent flux reporter, is blocked in the fly fat body (**Figure 2A-2D**).

3. “In Fig. 1J, is there statistically significant difference?”

The graph in Figure 1J represents the Ref(2)P immunoblot data shown in Figure 1I. This was the reproducible trend in all biological replicates, even though the quantification of the trend was not statistically significant (see below, Figure B).

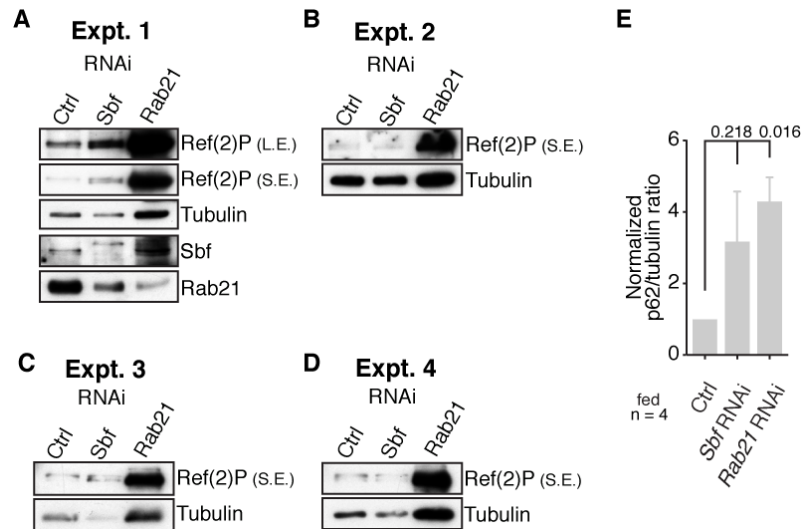


Figure B: Rab21 and Sbf are required for Ref(2)P autophagic clearance. (A-D) Ref(2)P protein levels in fat body lysates from four independent biological replicates in control or RNAi conditions, as shown, with long (L.E.) and short exposures (S.E.) from same blot. **(E)** Averaged ratio of Ref(2)P to tubulin integrated densities from four representative immunoblots in (A-D).

4. “Page 7, line 3: Figure numbers should be replaced (IH with 1H, IJ with 1J).”

Thank you for your careful reading of our manuscript and for catching this necessary edit! These numbers have been corrected.

5. “Page 7, line 5 from the bottom: "the mutant" should be substituted with "the knock-down".”

We agree, and this has been corrected as suggested.

6. “Page 8, line 2: the authors should discuss about not only increased number but also the size of autophagosome.”

We now extend our previous analysis of autophagosome area per cytoplasmic area (**Figure E1K**) to include an additional graph quantifying the average autophagosome area (**Figure E1L**) in control versus *Rab21* and *Sbf* RNAi conditions. This analysis demonstrates that autophagosome size is increased, and we now include this information in the text on **page 7**. We can only speculate that this could be a result of fusion between accumulated autophagosomes, or alternatively, an independent role for both *Sbf* and *Rab21* functions in regulation of autophagosome size.

7. “Page 8, subheading "Conserved human roles~" is not clear.”

We revised this subheading to read, “Conserved roles for human Rab21 and MTMR13 in autophagosome clearance.”

References:

Barr FA (2013) Rab GTPases and membrane identity: Causal or inconsequential? *J Cell Biol* **202**: 191-199

Furuta N, Fujita N, Noda T, Yoshimori T, Amano A (2010) Combinational soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins VAMP8 and Vti1b mediate fusion of antimicrobial and canonical autophagosomes with lysosomes. *Mol Biol Cell* **21**: 1001-1010

Ganley IG, Wong PM, Gammoh N, Jiang X (2011) Distinct autophagosomal-lysosomal fusion mechanism revealed by thapsigargin-induced autophagy arrest. *Mol Cell* **42**: 731-743

Itakura E, Kishi-Itakura C, Mizushima N (2012) The Hairpin-type Tail-Anchored SNARE Syntaxin 17 Targets to Autophagosomes for Fusion with Endosomes/Lysosomes. *Cell* **151**: 1256-1269

Jean S, Cox S, Schmidt EJ, Robinson FL, Kiger A (2012) Sbf/MTMR13 coordinates PI(3)P and Rab21 regulation in endocytic control of cellular remodeling. *Mol Biol Cell* **23**: 2723-2740

Jean S, Kiger AA (2012) Coordination between RAB GTPase and phosphoinositide regulation and functions. *Nat Rev Mol Cell Biol* **13**: 463-470

Juhász G, Hill JH, Yan Y, Sass M, Baehrecke EH, Backer JM, Neufeld TP (2008) The class III PI(3)K Vps34 promotes autophagy and endocytosis but not TOR signaling in *Drosophila*. *J Cell Biol* **181**: 655-666

Mizuno-Yamasaki E, Rivera-Molina F, Novick P (2012) GTPase networks in membrane traffic. *Annual Review of Biochemistry* **81**: 637-659

Moreau K, Ravikumar B, Renna M, Puri C, Rubinsztein DC (2011) Autophagosome Precursor Maturation Requires Homotypic Fusion. *Cell* **146**: 303-317

Pryor PR, Mullock BM, Bright NA, Lindsay MR, Gray SR, Richardson SCW, Stewart A, James DE, Piper RC, Luzio JP (2004) Combinatorial SNARE complexes with VAMP7 or VAMP8 define different late endocytic fusion events. *EMBO Reports* **5**: 590-595

Puri C, Renna M, Bento CF, Moreau K, Rubinsztein DC (2013) Diverse Autophagosome Membrane Sources Coalesce in Recycling Endosomes. *Cell* **154**: 1285-1299

Rusten TE, Lindmo K, Juhasz G, Sass M, Seglen PO, Brech A, Stenmark H (2004) Programmed autophagy in the Drosophila fat body is induced by ecdysone through regulation of the PI3K pathway. *Dev Cell* **7**: 179-192

Scott RC, Schuldiner O, Neufeld TP (2004) Role and regulation of starvation-induced autophagy in the Drosophila fat body. *Dev Cell* **7**: 167-178

Takats S, Nagy P, Varga A, Piracs K, Karpati M, Varga K, Kovacs AL, Hegedus K, Juhasz G (2013) Autophagosomal Syntaxin17-dependent lysosomal degradation maintains neuronal function in Drosophila. *J Cell Biol* **201**: 531-539

Yoshimura S, Gerondopoulos A, Linford A, Rigden DJ, Barr FA (2010) Family-wide characterization of the DENN domain Rab GDP-GTP exchange factors. *J Cell Biol* **191**: 367-381

Zhu G, Chen J, Liu J, Brunzelle JS, Huang B, Wakeham N, Terzyan S, Li X, Rao Z, Li G, Zhang XC (2007) Structure of the APPL1 BAR-PH domain and characterization of its interaction with Rab5. *EMBO J* **26**: 3484-3493

2nd Editorial Decision

02 January 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.