EMBO reports

Manuscript EMBOR-2014-38618

PI3P phosphatase activity is required for autophagosome maturation and autolysosome formation

Yanwei Wu, Shiya Cheng, Hongyu Zhao, Wei Zou, Sakako Yoshina, Shohei Mitani, Hong Zhang and Xiaochen Wang

Corresponding author: Xiaochen Wang, National Institute of Biologcal Sciences

Review timeline:

Submission date:	12 February 2014
Editorial Decision:	13 March 2014
Revision received:	21 June 2014
Editorial Decision:	09 July 2014
Revision received:	10 July 2014
Accepted:	11 July 2014

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: Nonia Pariente

1st Editorial Decision 13 March 2014

Thank you for your patience while your study has been under peer-review at EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, although referee 2 is more negative about the overall advance, referees 1 and 3 are supportive of the study. Nevertheless, all three raise various concerns that would need to be addressed.

Given that all three referees provide constructive suggestions on how to strengthen the study, I am happy to invite its revision. As the reports are below, I will not detail them here. However, it is clear that a more thorough quantification and statistical analyses are required throughout the manuscript, as well as more details on how the data was analyzed and represented. All concerns of all three referees (several of which are overlapping) should be fully addressed during revision. I am uncertain if autophagy flux assays are technically feasible in the worm; if not, you would obviously not need to provide these.

If the referee concerns are adequately addressed, we will be happy to accept your study for publication in EMBO reports. Please note that it is our policy to undergo one round of revision only

and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

Revised manuscript can contain up to five figures, which will give you more space to address the referee concerns. On the other hand, your text is longer than we can accommodate, and the referees are asking for additional discussions and inclusion of more references. The length of revised manuscripts must be a maximum of 30,000 characters (including spaces, figure legends and references), but shortening will be easier by combining the Results and Discussion into a single section, which we require, and which will help eliminate the redundancy that is inevitable when discussing the same experiments twice (in this case, I feel a large part of the current Discussion section could be removed). In addition, the introduction is quite lengthy and couls also be more succinctly summarized. Please note that basic Materials and Methods required for understanding the experiments performed must remain in the main text; however, additional detailed information necessary to reproduce them may be included as Supplementary Material.

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

Do not hesitate to get in touch with me if I can be of any help during the revision process. I am very much looking forward to receiving your revised study.

REFREE REPORTS:

Referee #1:

This manuscript by Wu et al reports the identification of worm myotubularin phosphatase MTM-3 as a positive regulator of autophagy. Specifically, the authors show that the 3-phosphatase activity of MTM-3 towards PI3P is involved in a late stage of autophagy (downstream of EPG-6 and upstream of EPG-5), where it facilitates the maturation of autophagosomes and their conversion into autophagolysosomes. This function is specific for MTM-3 as the other isoforms of the worm myotubularin family have no effects on autophagy. They also show that MTM-3 is recruited to autophagosomes by PI3P itself and that ablation of MTM-3 increases the recruitment (or prevents the dissociation) of PI3P effector ATG-18 to nascent autophagosomes. Altogether this report shows a positive role for MTM-3 and PI3P turnover in autophagosome maturation and autophagolysosome formation.

This is an interesting report that provides further evidence that PI3P turnover plays a positive role in autophagy. While this notion has already been put forward in a yeast study by Reggiori and colleagues focusing on the myotubularin ortholog, this report presents an important confirmation in a higher eukaryote, and supports it with a variety of elegant readouts specific for the worm model system. Overall, the work is both elegant and compelling, and suggests that the worm MTM-3 may be the ortholog of mammalian MTMR3. There are only a few points that need to be addressed prior to publication in this referee's view. These are as follows.

1. All the quantitative analyses need to include in the legend sample sizes, what the values denote, what the error bars are, etc...Also, statistical analyses are not performed systematically. For all the graphs showing % colocalization, there is no indication provided on the variability associated with these experiments. The authors could express this variability simply by comparing the % colocalization values are in different animals, so that a mean and a standard deviation can be calculated.

2. In Figure 2, the subcellular localization of the MTM3 mutant is very striking, but only a small fraction of the staining actually colocalizes with LGG-1 suggesting that the enzyme may not reside on the autophagosomes per se. Could it be localized on some endosomal compartment that contribute to autophagosomal cup expansion? In fact, could MTM3 act "in trans" by dephosphorylating PI3P on membranes it is not associated with? The authors should attempt to

resolve this.

3. In Figure 2G, there are quite a few LGG-1 puncta left in the Vps34 mutant worm. Does it suggest that autophagy can occur independently of Vps34, as recently reported in some mouse models?

4. In Figure 3, it seems that multiple single and double mutants are shown, but the wild type worms are missing.

5. Page 6: the authors should define the PGL granules.

6. Figure 1I-J-K (EM) could be considered a separate figure, considering it is cited at the end of the paper.

7. There are several recent and comprehensive reviews on lipids (or PI3P) and autophagy which the authors may want to cite in their manuscripts, considering they cannot cite all the original studies implicating PI3P in autophagy.

Referee #2:

In this manuscript Wu and co-workers identify C.elegans Myotubularin-3 (ceMTM3) as a regulator of autophagy, and show that its PtdIns3P phosphatase activity is required for autophagy. They show convincingly that ceMTM3 is recruited to autophagic membranes in a PtdIns3P-dependent manner and that human MTM3 can rescue the autophagy defect seen in mtm3 mutants. Loss of ceMTM3 leads to increased membrane localization of Atg18 in a PtdIns3P-dependent manner. The authors argue that MTM3 functions as a positive regulator at the late stage of autophagy, downstream of the ATG-2/EPG-6 complex and upstream of EPG-5 to promote autophagosome maturation into autolysosomes.

This study is general well performed and well written, but it does not meet the high standards set for publication in EMBO Reports when it comes to novelty and new mechanistic insight. Several recent studies have found that PtdIns3P de-phosphorylation by myotubularins regulate autophagy; MTMR3 and MTMR14/Jumpy as negative regulators of autophagosome formation (Vergne et al, EMBO J 2009; Taguchi-Atarashi et al, Traffic 2010) and yeast Ymr1 (Cebollero et al, Curr Biol, 2012) as a positive regulator of autophagosome completion (through dissociation of Atg proteins). It has also been found that knockdown of ceMTM3 by RNAi caused excessive autophagy in different tissues, leading to increased lysosomal activity and necrotic cell death (Yu X et al, BMC Cell Biol, 2012).
The authors argue that ceMTM3 is a positive regulator that promotes fusion of autophagosomes with lysosomes, but the data provided are not of sufficient quality to conclude this. Most of their results are based on quantification of confocal images of embryos stained with different markers, but there is no information (except for Fig. 4R) about the number of embryos used for quantification, how many independent experiments were done and how the quantifications were done (manual or automatic). Some graphs also lack error bars and significance values.
In figure 1 they include EM pictures showing an accumulation of autophagosomes in mtm3 mutants, but it is not possible to conclude from these pictures whether the autophagosomes are properly closed or not. Moreover, they show by western blot that LGG1-II (Atg8-II) accumulates in mtm3 mutants and conclude that autophagosome maturation is inhibited. In the absence of proper flux experiments (+/- lysosomal inhibitor) it is not possible to conclude whether autophagic flux is increased (due to more autophagosomes formed) or inhibited (due to defect autophagosome maturation, fusion with lysosomes). The authors suggest that the latter is the case (as they see accumulation of p62 and SEPA-1), and this reviewer agree that is likely the case, but they should anyway perform proper flux experiments. They try to address this issue in Figure 4 with laat-1 and cup-5 mutants (which affect lysosomal properties), but again they data are not convincing due to insufficient statistical information.
It is also not clear why they use colocalization and morphology of LGG1 and PGL as a read-out to pinpoint where in the pathway MTM3 works.

Minor comments:

Figure 1H: include an autophagy mutant for comparison.
Figure 3: should do same stainings in wild type for comparison. The authors should explain why their data with atg2 and atg18 mutants are so different, as these two proteins are supposed to act together.
Fig. 3L: does not make sense to write e.g. 28,7% in the text when there are no error bars in the graph.
Fig. S3T: there is no description of the methods used here.
Introduction (top p4); the authros state that "Although autophagosome biogenesis has been extensively studied, relatively little is known about the regulation of autophagosome maturation and autolysosome formation». This is not true and they should include some references to this work.
Right below (p4) they write "Upon autophagy induction, PtdIns3P is produced at the PAS in yeast or a subdomain of the ER or ER-mitochondria contact sites in higher eukaryotes and promotes autophagosome biogenesis through recruitment of PtdIns3P-binding effectors such as Atg18/WIPI and DFCP1 (Mizushima et al, 2011)". More references should be included here, especially Hamaski et al, Nature 2013 who showed autophagosome biogenesis from the ER-mitochondria contact sites.

Referee #3:

Wu et al report that the conserved myotubularin phosphatase MTM-3 plays a role in autophagy. Although myotubularin phosphatases have been previously linked to autophagy in metazoans, their role has been as a negative regulators of the initiation of autophagy. The authors here present a careful analysis of MTM-3 in C. elegans, and unveil that MTM-3 promotes autolysosome formation in basal conditions in nematodes. This work would be of interest to the readership of EMBO Reports. Having said that, I have a few concerns about some of the claims made in the manuscript and I would suggest a few additional experiments. Also, some experimental conditions would need to be described and some observations further discussed.

1) Although epistasis and mutational analyses suggest a role for MTM-3 in autophagy regulation through its lipolytic activity, the experiment presented in Figure S3T is the only experiment showing that MTM-3 directly modulates PI3P levels. Given that this is central to the model put forward by the authors, this reviewer would recommend Figure S3T to be moved to the main body of the manuscript. In addition, a more detailed description of the experimental conditions is necessary. Was a purified tagged MTM-3 tested? Which one? Which concentration? Which substrate? Which reaction conditions?

2) It is possible that, as part of a compensatory response, MTM-3(C459S)::GFP is expressed at higher levels than MTM-3::GFP. Excessive MTM-3(C459S)::GFP could promote unspecific protein localization to the autophagosomal compartment. A comparison of the levels of expression of MTM-3::GFP and MTM-3(C459S)::GFP by western blot is necessary.

3) Following a similar reasoning, authors should test the levels of expression (Western blot) of ATG-18::GFP in wild-type and mtm-3 animals. Also, the difference in punctae number between Figure 4J and 4I, K-P is not striking; quantification of ATG-18::GFP signal from at least 10 wild type and mtm-3 embryos should be presented.

4) Describe if mtm deletion mutants other than mtm-3 are expected to be nulls (does the deletion span the phosphatase domain? Other essential regions?). Alternatively, were the other mtm genes inactivated by injected dsRNA? This is relevant to understand if functional analysis of mtm-1, 5-, 6, and 9 was as exhaustive as the one made for mtm-3.

Minor points
1) Figure 2H: add alleles to figure legend.

2) Figure 2I: explain normalization and lack of statistical analysis.

3) The observation that MTM-3 overexpression does not inhibit autophagy calls for a discussion about tight control of the initiation of autophagy but not of autolysosome formation. Alternatively,

limiting MTM-3 substrate or other rate limiting factors could explain the lack of an obvious MTM-3 overexpression phenotype. These and/or other hypothesis should be discussed.

4) The authors should provide an explanation (hypothesis) for the homogeneous distribution of MTM-3(C459S)::GFP in vps-34 animals. Does this suggest that in the presence of a functional endo/lysosomal machinery MTM-3 is trafficked through the endo/lysosomal compartment and consequently in vps-34 animals MTM-3 is disperse in the cytoplasm?

5) Figure legend for Figure 3L and 4H should clarify that wild-type values are not shown because SEPA or SQST-1 punctae are hardly visible in wild-type animals.

6) Readers would greatly benefit from a comparative model of autophagy (yeast, worms, mammals) highlighting the steps at which PI3P kinases and phosphatases act.

7) Authors should discuss negative role of MTM-3 in mammals and positive role in C. elegans (even when the human protein was expressed in C. elegans). Are there unknown partners that tune the function of MTM-3 according to cellular needs?

1st Revision - authors' response 21 June 2014

The major changes that we made in the revised manuscript are as follows:

1. As suggested by all three reviewers, all quantification analyses were re-performed in at least 10 animals and error bars and statistical analyses were included in all assays. We also included information about sample size and statistical analysis in the figure legends and described how quantification analyses were performed in the Methods.
2. We have moved the EM data from Figure 1 to Figure 4 as suggested by reviewer 1, and we moved the data on phosphatase activity of MTM-3 from Figure S3 to Figure 2 as suggested by reviewers 2 and 3. A detailed description of the experimental conditions of the phosphatase activity assay is also included in the Methods.
3. As suggested by reviewer 3, we have examined expression levels of GFP::MTM-3, GFP::MTM-3(C459S) and ATG-18::GFP by western blot.
4. As suggested by reviewers 3, we have discussed the MTM-3 overexpression phenotype and the distinct effects of worm/yeast and mammalian myotubularin phosphatases on autophagy.
5. We have included additional references as suggested by reviewers 1 and 2.

Our point-by-point responses to the reviewers' comments are listed below:

Reviewer 1

1. All the quantitative analyses need to include in the legend sample sizes, what the values denote, what the error bars are, etc...Also, statistical analyses are not performed systematically. For all the graphs showing % colocalization, there is no indication provided on the variability associated with these experiments. The authors could express this variability simply by comparing the % colocalization values are in different animals, so that a mean and a standard deviation can be calculated.

As suggested by the reviewer, we have re-performed all quantification analyses with at least 10 embryos scored in each strain. The information about sample size and statistical analysis (error bars and P value) is now included in the figure legends. A detailed description of the quantification analyses is also included in the Methods in the revised manuscript.

2. In Figure 2, the subcellular localization of the MTM3 mutant is very striking, but only a small fraction of the staining actually colocalizes with LGG-1 suggesting that the enzyme may not reside on the autophagosomes per se. Could it be localized on some endosomal compartment that contribute to autophagosomal cup expansion? In fact, could MTM3 act "in trans" by dephosphorylating PI3P on membranes it is not associated with? The authors should attempt to resolve this.

In wild-type, *mtm-3(tm4475)* or *atg-2(bp576)* embryos, over 60% of GFP::MTM-3(C459S)-positive puncta co-localize with LGG-1, suggesting that the majority of the MTM-3(C459S) resides on autophagic structures. To examine whether MTM-3(C459S) may localize to endosomal compartments, we inactivated *rab-5* and *rab-7* by RNAi, which affects endosome formation. We found that MTM-3(C459S) puncta were not affected by *rab-5* or *rab-7* RNAi, suggesting that they do not localize to endosomes. We have included these data in the revised manuscript (Figure 2H).

3. In Figure 2G, there are quite a few LGG-1 puncta left in the Vps34 mutant worm. Does it suggest that autophagy can occur independently of Vps34, as recently reported in some mouse models?

As pointed out by the reviewer, we found that LGG-1 puncta were not completely eliminated in *vps-34(h797)* mutants. The *vps-34(h797)* mutant is lethal and therefore maintained as *dpy-5(e61) vps-34(h797)*; *qxEx* [*vps-34(+)*; $P_{sur\text{-}5}$SUR-5-GFP]. The non-green progeny were scored as *vps-34(lf)* [*dpy-5(e61) vps-34(h797)*]. It is possible that residual *vps-34* activity still remains in the non-green embryos due to the transgene expression in the previous generation. However, we cannot firmly exclude the possibility that these LGG-1 puncta represent the occurrence of VPS-34-independent autophagy.

4. In Figure 3, it seems that multiple single and double mutants are shown, but the wild type worms are missing.

We apologize for not explaining this more clearly in the original manuscript. The SEPA-1 aggregates are not visible in wild-type embryos at the stage that we examined. Therefore, wild-type images are not included. We have explained this in the Figure legend in the revised manuscript.

5. Page 6: the authors should define the PGL granules.

We have defined the PGL granules in the text as suggested by the reviewer (Page 5).

6. Figure 1I-J-K (EM) could be considered a separate figure, considering it is cited at the end of the paper.

As suggested by the reviewer, we have moved the EM data from Figure 1 to Figure 4 where other data regarding autolysosome formation are shown.

7. There are several recent and comprehensive reviews on lipids (or PI3P) and autophagy which the authors may want to cite in their manuscripts, considering they cannot cite all the original studies implicating PI3P in autophagy.
We have included recent reviews regarding the role of lipids (or PtdIns3P) in autophagy as suggested by the reviewer.

We sincerely thank reviewer 1 for his/her very helpful and constructive comments and suggestions.

Reviewer 2

1. The authors argue that ceMTM3 is a positive regulator that promotes fusion of autophagosomes with lysosomes, but the data provided are not of sufficient quality to conclude this. Most of their results are based on quantification of confocal images of embryos stained with different markers, but there is no information (except for Fig. 4R) about the number of embryos used for quantification, how many independent experiments were done and how the quantifications were done (manual or automatic). Some graphs also lack error bars and significance values.

To address the concern raised by the reviewer, we have re-performed all quantitative analyses with at least 10 embryos scored in each strain. The error bars and significance values are included in all assays. In the revised manuscript, we have included a detailed description of how the quantitative analyses are performed (main text) and how the statistical analyses are done (supplemental information).

2. In figure 1 they include EM pictures showing an accumulation of autophagosomes in mtm3 mutants, but it is not possible to conclude from these pictures whether the autophagosomes are

properly closed or not. Moreover, they show by western blot that LGG1-II (Atg8-II) accumulates in mtm3 mutants and conclude that autophagosome maturation is inhibited. In the absence of proper flux experiments (+/- lysosomal inhibitor) it is not possible to conclude whether autophagic flux is increased (due to more autophagosomes formed) or inhibited (due to defect autophagosome maturation, fusion with lysosomes). The authors suggest that the latter is the case (as they see accumulation of p62 and SEPA-1), and this reviewer agree that is likely the case, but they should anyway perform proper flux experiments. They try to address this issue in Figure 4 with laat-1 and cup-5 mutants (which affect lysosomal properties), but again they data are not convincing due to insufficient statistical information.

We agree with the reviewer that it is ideal if autophagic flux can be measured. Unfortunately, this assay is technically not feasible in *C. elegans* embryos. In *C. elegans* embryos where the basal level of autophagy is examined, cell division occurs very fast and embryonic cells undergo rapid movements during morphogenesis. It is impossible to synchronize embryonic cells to the same developmental stage. In addition, multiple cell types exist in the embryo which may show distinct autophagy responses. The autophagy activity, which is indicated by the number of LGG-1/LC3 or SQST-1 aggregates, however, cannot be measured and compared at the single cell level in *C. elegans* embryos. Nevertheless, as pointed out by the reviewer, our genetic and cell biological data strongly support that MTM-3 is a positive regulator of autophagy and loss of MTM-3 impairs autophagy. 1) Loss of *mtm-3* causes accumulation of multiple autophagic cargoes including SEPA-1 and SQST-1. 2) *mtm-3* mutants contain significantly increased LGG-1/LC3 puncta and accumulate both unprocessed and lipid-conjugated LGG-1, suggesting a defect in the autophagy process. 3) Like other autophagy-defective mutants such as *epg-6 (lf)*, loss of *mtm-3* significantly affects the survival of newly hatched L1 larvae in the absence of food, a process that absolutely requires autophagy activity. 4) Our epistatic analyses have placed MTM-3 in the aggrephagy pathway at the step downstream of ATG-2-EPG-6 and upstream of EPG-5 and CUP-5. 5) We found that autophagosomes persisted in *mtm-3(lf)* mutants and are separable from lysosomes. Altogether, these data indicate that MTM-3 acts to promote autophagosome maturation into autolysosomes. To address the concern regarding insufficient statistical information in Figure 4, we re-performed all quantitative analyses with at least 10 embryos scored in each strain and data shown as mean ± SD. Student's two way unpaired *t*-test was performed and P values are indicated (Figure 4H, I in the revised manuscript).

3. It is also not clear why they use colocalization and morphology of LGG1 and PGL as a read-out to pinpoint where in the pathway MTM3 works.

Studies in Hong Zhang's lab have revealed that a variety of protein aggregates are removed by the basal level of autophagy during *C. elegans* embryogenesis, including the germline P granule

components PGL-1 and PGL-3, namely PGL granules, and the *C. elegans* p62 homolog SQST-1 (Zhang Y.X. et al., Cell 136, 308-21, 2009; Tian Y. et al., Cell 141, 1042-55, 2010). This process, which is referred to as aggrephagy, has then been employed by the Zhang lab to identify and characterize essential autophagy genes. They found that different autophagy mutants exhibit distinct phenotypes including the formation, morphology and distribution of PGL granules, SQST-1 aggregates and LGG-1 puncta. On the basis of these genetic phenotypes in various single and double mutants, the genetic hierarchical relationship of autophagy genes in the aggrephagy pathway was established (reviewed by Lu Q, et al., Biochem. J. 452, 381-90, 2013). Employing the same approach, we found in this study that MTM-3 acts at the step downstream of ATG-2-EPG-6 and upstream of EPG-5 and CUP-5 in the aggrephagy pathway.

4. Minor comments:

1) Figure 1H: include an autophagy mutant for comparison.

As suggested by the reviewer, we have included *epg-6* mutants in Figure 1H for comparison. *mtm-3* and *epg-6* mutants show very similar phenotypes in the survival of L1 larvae under starvation condition, with a mean life span of 3.5 days in *mtm-3(lf)* and 4 days in *epg-6(lf)*.

2) Figure 3: should do same stainings in wild type for comparison. The authors should explain why their data with atg2 and atg18 mutants are so different, as these two proteins are supposed to act together.

We apologize for not explaining this more clearly in the original manuscript. The SEPA-1 aggregates are not visible in wild-type embryos at the stage that we examined. Therefore, wild-type images are not included. We have explained this in the Figure legend in the revised manuscript. Regarding the role of ATG-2 and ATG-18, it has been shown by the Zhang lab that in *C. elegans*, EPG-6, a WIPI3/4 homolog, but not ATG-18 which is related to WIPI1/2, interacts directly with ATG-2, suggesting that EPG-6/WIPI4 is the functional orthologue of yeast Atg18 (Lu Q. et al., Dev. Cell 21, 1-15, 2011). Consistent with this, *epg-6* and *atg-2* mutants exhibit the same phenotypes, while *atg-18* mutants show distinct phenotypes from *atg-2* and *epg-6* (Lu Q. et al., Dev. Cell 21, 343-57, 2011). The distinct steps at which ATG-18 and EPG-6/ATG-2 act in the aggrephagy has been explained in the revised manuscript (Page 8).

3) Fig. 3L: does not make sense to write e.g. 28,7% in the text when there are no error bars in the graph.

The quantitative analyses have been re-performed in at least 10 embryos and data are shown as mean±SD. The statistical analyses were also performed and P values are indicated.

4) Fig. S3T: there is no description of the methods used here.

In the revised manuscript, we have included a detailed protocol by which the phosphatase activity of MTM-3 was measured (Page 13).

5) Introduction (top p4); the authros state that "Although autophagosome biogenesis has been extensively studied, relatively little is known about the regulation of autophagosome maturation and autolysosome formation». This is not true and they should include some references to this work.

We have rephrased the statement and included additional references as suggested by the reviewer.

6) Right below (p4) they write "Upon autophagy induction, PtdIns3P is produced at the PAS in yeast or a subdomain of the ER or ER-mitochondria contact sites in higher eukaryotes and promotes autophagosome biogenesis through recruitment of PtdIns3P-binding effectors such as Atg18/WIPI and DFCP1 (Mizushima et al, 2011)". More references should be included here, especially Hamaski et al, Nature 2013 who showed autophagosome biogenesis from the ER-mitochondria contact sites.

We have included more references including Hamaski et al., Nature 2013 as suggested by the reviewer.

We sincerely thank reviewer 2 for his/her very helpful and constructive comments and suggestions.

Reviewer 3

1. Although epistasis and mutational analyses suggest a role for MTM-3 in autophagy regulation through its lipolytic activity, the experiment presented in Figure S3T is the only experiment showing that MTM-3 directly modulates PI3P levels. Given that this is central to the model put forward by the authors, this reviewer would recommend Figure S3T to be moved to the main body of the manuscript. In addition, a more detailed description of the experimental conditions is necessary. Was a purified tagged MTM-3 tested? Which one? Which concentration? Which substrate? Which reaction conditions?

We apologize for not explaining the experimental conditions and results more clearly in the original manuscript and thank the reviewer for this very good suggestion. As suggested by the reviewer, we have moved the phosphatase activity assay from Figure S3T to Figure 2J in the revised manuscript and included a detailed protocol by which the phosphatase activity of MTM-3 was measured (Page 13).

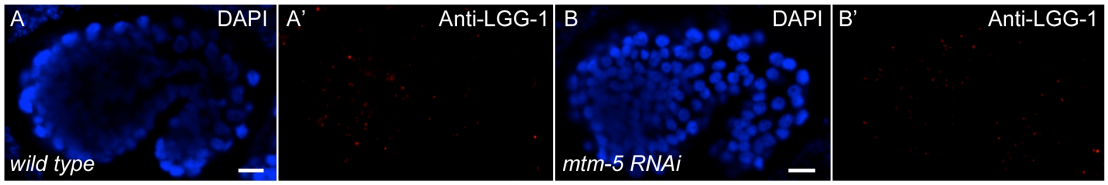
2. It is possible that, as part of a compensatory response, MTM-3(C459S)::GFP is expressed at higher levels than MTM-3::GFP. Excessive MTM-3(C459S)::GFP could promote unspecific

protein localization to the autophagosomal compartment. A comparison of the levels of expression of MTM-3::GFP and MTM-3(C459S)::GFP by western blot is necessary.
As suggested by the reviewer, we examined expression levels of GFP::MTM-3 and GFP::MTM-3(C459S) by western blot analysis using anti-GFP antibodies. Indeed, GFP::MTM-3(C459S) is expressed at a higher level than the wild-type protein (GFP::MTM-3), which may be caused by a compensatory response as suggested by the reviewer. However, we found that loss of VPS-34, the PI3 kinases, but not ATG-2, which regulates the progression from isolation membrane to autophagosome, greatly reduced the number of GFP::MTM-3(C459S) puncta, and co-localization of GFP::MTM-3(C459S) with LGG-1 (14.9% in *vps-34* vs 66.8% in wild type). Moreover, expression of GFP::MTM-3(C459S, del PH-GRAM), which lacks the phosphinositide-binding PH-GRAM domain of MTM-3, failed to form puncta (Figure 2C). These results strongly suggest that GFP::MTM-3(C459S) associates with autophagic structures specifically and this association is regulated by PtdIns3P. We have included the western blot analysis of GFP::MTM-3 and GFP::MTM-3(C459S) in Figure S3 in the revised manuscript.

3. Following a similar reasoning, authors should test the levels of expression (Western blot) of ATG-18::GFP in wild-type and mtm-3 animals. Also, the difference in punctae number between Figure 4J and 4I, K-P is not striking; quantification of ATG-18::GFP signal from at least 10 wild type and mtm-3 embryos should be presented.
As suggested by the reviewer, we performed western blot analysis to examine expression levels of ATG-18::GFP in wild-type and *mtm-3(lf)* worms. We found that ATG-18::GFP is expressed at a higher level in wild type than in *mtm-3 (lf)* mutants, suggesting that the increased number of ATG-18::GFP puncta formed in *mtm-3* is not caused by high levels of expression. To quantify the ATG-18::GFP puncta in wild type, *mtm-3*, *mtm-3;lgg-1* RNAi and *mtm-3;vps-34* RNAi, 15 embryos from each strain were scored. In *mtm-3(tm4475)* embryos, an average of 24.5±4.8 ATG-18::GFP puncta were observed, which is significantly higher than in wild type (0.8±0.7). The number of ATG-18::GFP puncta was greatly reduced in *mtm-3;lgg-1* RNAi (4.4±1.9) and *mtm-3;vps-34* RNAi worms (6.8±3.3), indicating that ATG-18::GFP associates with autophagic structures in a PtdIns3P-dependent manner. These data are presented in the new Figure 5 in the revised manuscript.

4. Describe if mtm deletion mutants other than mtm-3 are expected to be nulls (does the deletion span the phosphatase domain? Other essential regions?). Alternatively, were the other mtm genes inactivated by injected dsRNA? This is relevant to understand if functional analysis of mtm-1, 5-, 6, and 9 was as exhaustive as the one made for mtm-3.

The *ok742* allele of *mtm-1* contains a 1314 bp deletion that removes the region from intron 4 to exon 7 of the *mtm-1* gene, resulting in MTM-1 truncation containing only the N-terminal PH-GRAM domain. It is likely a null mutation of *mtm-1,* which causes embryonic lethality and larval arrest. The deletion alleles of *mtm-6 and mtm-9* , *ok330* and *ok3523*, contain a 1235 bp and a 635 bp deletion, respectively, and cause protein truncations that remove the majority of the catalytic phosphatase domain of MTM-6 and 9. Therefore, these two alleles are very likely to be strong loss-of-function or null mutations of *mtm-6* and *mtm-9*. The *ok469* allele of *mtm-5* contains a 665 bp deletion that removes the region from exon 7 to 9, resulting in a truncated MTM-5 protein lacking the conserved myotubularin domain. Given that the phosphatase domain of MTM-5 is not affected in *ok469*, RNAi was performed to inactive *mtm-5*. We found that like *mtm-5(ok469)*, inactivation of *mtm-5* by RNAi did not cause accumulation of LGG-1 (see figure below), confirming that loss of *mtm-5* does not affect autophagy. We have included a detailed description of *mtm* deletions in the revised manuscript (Methods in supplemental information).



5. Minor points

1) Figure 2H: add alleles to figure legend.

We have included allele names in the figure legend as suggested by the reviewer.

2) Figure 2I: explain normalization and lack of statistical analysis.

We have re-performed all quantification analyses including that shown in Figure 2I. Statistical analysis was performed and P values are shown. A detailed description of how the quantitative analyses are performed is also included in the revised manuscript (Page 12-13).

3) The observation that MTM-3 overexpression does not inhibit autophagy calls for a discussion about tight control of the initiation of autophagy but not of autolysosome formation. Alternatively, limiting MTM-3 substrate or other rate limiting factors could explain the lack of an obvious MTM-3 overexpression phenotype. These and/or other hypothesis should be discussed.

We thank the reviewer for this very good suggestion. As pointed out by the reviewer, we think that the lack of an autophagy phenotype in worms that overexpress MTM-3 suggests involvement of

rate-limiting regulatory factors. These rate-limiting factors may regulate recruitment, release or activity of MTM-3 on autophagic structures. Consistent with this notion, we observed that expression of MTM-3(C459S), a catalytically inactive substrate-trapping form of MTM-3, but not wild-type MTM-3, displays an autophagic localization pattern. This suggests that autophagic recruitment and/or release of MTM-3 may be tightly regulated. We have discussed this point in the revised manuscript (Page 12).

4) The authors should provide an explanation (hypothesis) for the homogeneous distribution of MTM-3(C459S)::GFP in vps-34 animals. Does this suggest that in the presence of a functional endo/lysosomal machinery MTM-3 is trafficked through the endo/lysosomal compartment and consequently in vps-34 animals MTM-3 is disperse in the cytoplasm?

We showed previously that loss of VPS-34 causes significantly reduced PtdIns3P on autophagic structures (Cheng et al., Autophagy 9(12):2022-32, 2013). Here we found that loss of *vps-34*, which affects PtdIns3P generation on autophagic compartments, significantly reduced the number of GFP::MTM-3(C459S) puncta and its co-localization with LGG-1, suggesting that MTM-3(C459S) associates with autophagic structures in a PtdIns3P-dependent manner. Given that GFP::MTM-3 and GFP::MTM-3(C459S, del PH-G), which lacks the phosphoinositide-binding domain of MTM-3, are diffuse in the cytoplasm in wild-type embryos, it is possible that MTM-3 is recruited from the cytoplasm to autophagic structures by PtdIns3P. Loss of *vps-34* affects PtdIns3P generation, causing cytoplasmic accumulation of MTM-3(C459S).

5) Figure legend for Figure 3L and 4H should clarify that wild-type values are not shown because SEPA or SQST-1 punctae are hardly visible in wild-type animals.

We have clarified this point in the legends of figures 3 and 4 in the revised manuscript.

6) Readers would greatly benefit from a comparative model of autophagy (yeast, worms, mammals) highlighting the steps at which PI3P kinases and phosphatases act.

As suggested by the reviewer, we have included a model that describes the role of MTM-3 in autophagy in the revised manuscript (Figure S8F). As a comparison, models of yeast and mammalian myotubularin phosphatases in autophagy are also included.

7) Authors should discuss negative role of MTM-3 in mammals and positive role in C. elegans (even when the human protein was expressed in C. elegans). Are there unknown partners that

tune the function of MTM-3 according to cellular needs?

The worm and yeast myotubularin phosphatases promote autolysosome formation, while mammalian Jumpy and MTMR3 negatively regulate autophagy initiation. This discrepancy raises the possibility that PtdIns3P turnover occurring at different stages may have distinct effects on autophagy. It is possible that MTM-3/Ymr1 and MTMR3/Jumpy are tightly regulated temporally and spatially by unknown factors, so that they are recruited to autophagic structures at a specific stage to control PtdIns3P turnover. We have discussed this point in the revised manuscript (Page 12).

We sincerely thank reviewer 3 for his/her very helpful and constructive comments and suggestions.

2nd Editorial Decision 09 July 2014

Thank you for your patience while your revised manuscript has been under peer-review. As you will see from the reports below, all referees are now positive about its publication in EMBO reports. I am thus very pleased to write with an 'accept in principle' decision, which means that we will accept your manuscript for publication once a few minor formatting issues/corrections have been addressed, as follows.

- Please make sure that the information regarding statistical analysis is included in the main Materials and Methods section. This section looks rather succinct. Please note that basic Materials and Methods required for understanding the experiments performed must remain in the main text, although additional details can be supplementary.

- The references need reformatting to EMBO reports style.

- We now encourage the publication of original source data -particularly for electrophoretic gels and blots, but also for graphs- with the aim of making primary data more accessible and transparent to the reader. If you agree, you would need to provide one PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures and an Excel sheet or similar with the data behind the graphs. The files should be labeled with the appropriate figure/panel number, and the gels should have molecular weight markers; further annotation could be useful but is not essential. The source files will be published online with the article as supplementary "Source Data" files and should be uploaded when you submit your final version. If you have any questions regarding this please contact me.

- As a standard procedure, we edit the title and abstract of manuscripts to make them more accessible to a general readership. Please find the edited versions below my signature and let me know if you do NOT agree with any of the changes.

- Lastly, every EMBO reports paper now includes a 'Synopsis' to further enhance its discoverability. Synopses are displayed on the html version and they are freely accessible to all readers. The synopsis includes a short standfirst text -I have added my proposal for this text below- as well as 2-3 one sentence bullet points that summarise the paper. These should be complementary to the abstract -i.e. not repeat the same text. This is a good place to include, as appropriate, key acronyms and

quantitative and organism (yeast, mammalian cells, etc) information. This synopsis will be accompanied by a graphic that reproduces the scheme you provided in Expanded figure 8, which will therefore be more visible in the main paper (only in the html). Could you supply the bullet points to accompany the standfirst? Do let me know if you would like to modify the standfirst blurb:

"The PI3P phosphatase MTM-3 is required for autophagosome maturation and autolysosome formation in C. elegans, highlighting the importance of reducing PI3P levels at the autophagosomal membrane during these late steps of autophagy.

2-3 bullet points"

After all remaining corrections have been attended to, you will receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thanks very much you for your contribution to EMBO reports.

****

Edited title and abstract

PI3P phosphatase activity is required for autophagosome maturation and autolysosome formation
Autophagosome formation is promoted by the PI3 kinase complex, and negatively regulated by myotubularin phosphatases, indicating that regulation of local phosphatidylinositol-3-phosphate (PtdIns3P) levels is important for this early phase of autophagy. Here, we show that the Caenorhabditis elegans myotubularin phosphatase MTM-3 catalyzes PtdIns3P turnover late in autophagy. MTM-3 acts downstream of the ATG-2/EPG-6 complex and upstream of EPG-5 to promote autophagosome maturation into autolysosomes. MTM-3 is recruited to autophagosomes by PtdIns3P and loss of MTM-3 causes increased autophagic association of ATG-18 in a PtdIns3P-dependent manner. Our data reveal critical roles of PtdIns3P turnover in autophagosome maturation and/or autolysosome formation.

****

REFEREE REPORTS:

Referee #1:

The authors have addressed all my concerns and issues and I thus recommend publication of their manuscript in its current form.

Referee #2:

In this revised manuscript the authors have addressed all my initial comments and concerns.

Referee #3:

The authors have adequately addressed my concerns

2nd Revision - authors' response 10 July 2014

Thank you very much for handling our manuscript. I am very happy to know that our manuscript is in principle accepted. We have made changes to address the formatting issues/corrections as suggested.

1. We agree with the edited title and abstract.
2. We have added 2 bullet points in the "Abstract" page.
3. We have reformatted the references to EMBO reports style.
4. We have included information regarding statistical analysis and immunostaining in the main Materials and Methods section, and removed these two parts from the Expanded View.
5. We provided an Excel sheet that contains the original quantification data behind the graphs.
6. We provided a PDF file that contains the original scan of the western blot analysis shown in Figure 1G.

Thanks again for your time and effort in handling our manuscript.

3rd Editorial Decision 11 July 2014

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. It has been a pleasure to serve as editor for your study.

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.

