LAPTM4B is a PtdIns(4,5)P2 Effector that Regulates EGFR Signaling, Lysosomal Sorting and Degradation

Xiaojun Tan, Yue Sun, Andrew C. Hedman, Narendra Thapa and Richard A. Anderson

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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.)

Editor: Andrea Leibfried

1st Editorial Decision 07 August 2014

Thank you for submitting your manuscript entitled 'LAPTM4B is a PtdIns(4,5)P2 Effector that Regulates EGFR Signaling, Lysosomal Sorting and Degradation'. I have now received the reports from all referees.

As you can see below, all referees find your manuscript potentially interesting. However, they raise various concerns and find that your conclusions are currently not sufficiently supported by the data provided. More specifically, the PIP2 binding to LAPTM4B has to be better supported and more insight into the LAPTM4B-Nedd4-Hrs interaction and the role of SNX5 in this context are needed. Referee #2 and #3 also raise various concerns regarding the quantitative imaging analysis of both EGFR trafficking and co-localization assays, which need to be resolved to allow further consideration here.

Given the clear comments provided, I would like to invite you to submit a revised version of the manuscript, addressing all concerns of the referees. Please contact me in case of further questions. I should also add that it is EMBO Journal policy to allow only a single round of revision and that it is therefore important to address all concerns raised at this stage.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.
REFEREE COMMENTS

Referee #1:

This manuscript describes a novel function for the cancer biomarker LAPTM4B as negative regulator of endosomal sorting of EGFRs. The authors identified LAPTM4B as an interactor of the endosomal PIP kinase PIPKIγ5. They found that its overexpression inhibited EGF-induced endosomal sorting and lysosomal degradation of EGFRs whereas its depletion had the opposite effects. Mechanistically, it was found that LAPTM4B promotes ubiquitination of the ESCRT-0 subunit Hrs, a condition previously reported to attenuate the activity of Hrs, apparently by promoting interaction between Hrs and the E3 ubiquitin ligase Nedd4. The authors also found that the basic N-terminus of LAPTM4B interacts with various phospholipids and that this was abolished by mutation of 6 arginine residues to glutamine. Interestingly, the 6RQ mutant was found to inhibit EGFR degradation even stronger than wild-type LAPTM4B, and the authors suggest that PI(4,5)P2, generated by PIPKIγ5, serves as negative regulator of LAPTM4B.

Overall this study contains excellent imaging and biochemical data, which are well supported by quantifications. Given the interest for LAPTM4B and EGFR in cancer, the present story should be of significant interest.

Major point:

The idea that LAPTM4B binds PI(4,5)P2, and that this is of functional importance, is not well supported by the lipid blot in Fig. 5F. Here, the strongest binding of LAPTM4B to PE, PI3P and PI5P whereas binding to PI(4,5)P2 is barely detectable. The ability of PI(4,5)P2 to stimulate the LAPTM4B-PIKIγ5 interaction could therefore be different from the one proposed. Because PI(4,5)P2 binding is central in the proposed model, the authors should investigate further the binding between LAPTM4B and phospholipids using alternative assays.

Minor point:

Since Nedd4 is thought to bind directly to Hrs, it is not evident how LAPTM4B would enhance this interaction. Any further insight on this would be helpful.

Referee #2:

In this work the impact of LAPTM4B on EGFR endosomal sorting and degradation is investigated. A number of solid and straightforward experiments are conducted that cogently demonstrate that LAPTM4B is able to inhibit EGFR sorting into intraluminal vesicles (ILVs) of late endosomes/MVBs. Importantly, this function is inhibited by binding of LAPTM4B to PtdIns(4,5)P2 and PIPKIγ5, unveiling a fine-tuned regulation of the process. Interestingly, within cells population the authors were able to correlate high levels of LAPTM4B to the inhibition of EGFR sorting into ILVs in normal growing conditions, in absence of perturbations. Molecularly, the authors showed that LAPTM4B could facilitate Nedd4-Hrs binding, thus increasing Hrs ubiquitination. As a consequence, they suggest that Hrs is inhibited (via an intramolecular interaction between Ub and ubiquitin-interacting motif within the Hrs moiety), thus not any more able to recruit the EGFR for sorting and degradation into the late endosomes/MVBs.

Although this final model is not completely conclusive, the work is straightforward and the experiments are very well controlled and of technical high quality. The mechanism of LAPTM4B regulation by PtdIns(4,5)P2 and PIPKIγ5 is deeply dissected. Finally, the findings are novel and clarify how EGFR fate and signaling is regulated by LAPTM4B in physiological conditions; they may also help to better understand the basis for LAPTM4B overexpression in cancer. For this reasons I suggest publication in EMBO after minor revision.

There are two points that need to be strengthened:
1) One critical issue is what is the fate of the EGFR that is not targeted to the intraluminal vesicles of MVBs due to inhibition by LAPTM4B. Authors measured EGFR recycling in a very indirect and not quantitative way, which actually cannot discriminate between alterations in recycling from differences in internalization rates. Authors need to provide a more quantitative and reliable assay to measure EGFR recycling, e.g. based on radioactive EGF ligand and/or FACS-based methods using EGFR antibodies to follow recycling of the receptor (see, for instance, Sorkin and Duex 2010; Sigismund et al., 2008).

2) A second issue concerns the mechanism of action of LAPTM4B on Nedd4 and Hrs ubiquitination. First, authors should provide more controls for the Hrs ubiquitination assay. Indeed, authors overexpress His-Ubiquitin but they did not show any western blot (WB) to control for equal level of transfection in the different samples. This control is mandatory. In addition, authors should confirm ubiquitination of Hrs in the opposite way, by immunoprecipitating Myc-Hrs and blotting anti-Ub (anti-His might be very dirty in WB, while for Ub different commercially available antibodies exist that work well in WB, e.g. P4D1 from Santa Cruz or FK2 from BIOMOL). Finally, in previous reports it was shown that LAPTM4B possesses PY motifs through which it can bind to Nedd4 (Milkereit and Rotin, 2011). This mechanism is not contemplated by the authors. However, it would be important to show that LAPTM4B mutant in PY motifs abolishes its binding to Nedd4 and, as a consequence, revert LAPTM4B phenotypes (i.e. increased Hrs ubiquitination and decreased EGFR degradation). This experiment would provide a final prove that LAPTM4B action on EGFR is via Hrs ubiquitination. Since Hrs has no PY motifs, one intriguing possibility -that can be discussed - might be that LAPTM4B serves as an adaptor/scaffold between Hrs and Nedd4, as shown for ARTs adaptors in Yeast and, more recently, for ARRDC3 in mammals (Nabhan et al., 2010).

Minor points:

Figure 2F. It is not clear what is required for. Authors compared two doses after different pulse and chase times. What are the conclusions? I would remove this panel (or put in the supplementary) since it does not add any valuable information.

Supplementary Figure S5 C-F. Authors try to show that LAPTM4B-dependent mechanism is specific for EGFR, while not applying to other receptors. However, data do not support this conclusion. In addition authors cite literature in support of the idea that degradation of c-Met does not require Hrs. However there are reports show the opposite (e.g. Hammond et al., 2003; Row et al., 2005; Abella et al., 2005). I strongly suggest to remove this part, which in my opinion does not add anything to the final message of the paper.

Referee #3:

In this manuscript Tan et al. describe a novel function to the lysosomal protein, LAMTM4B, namely, the regulation of intraluminal sorting of the activated EGF receptor (EGFR). The authors show that LAMTM4B overexpression delays the degradation of EGF receptors and prolongs its signaling activity. Conversely, LAMTM4B depletion accelerated the termination of EGF signaling and EGFR degradation. The authors also demonstrated a direct interaction between PIPKγ1 isoform 5 and LAMTM4B N-terminus that appears to be regulated by PI(4,5)P2. They showed that the membrane-adjacent polybasic domain of the LAMTM4B at the N-terminus associates with endosomal PtdIns(4,5)P2, which stabilizes the PIPKγ5-LAMTM4B interaction. To explain the mechanism of the LAMTM4B effect on EGF sorting, the authors showed that LAMTM4B acts as a negative regulator of Hrs-driven EGFR intraluminal sorting at the MVB. This is mediated by the interaction of LAMTM4B with Nedd4 ubiquitin ligase with enhanced ubiquitination of Hrs, thus inhibiting its interaction with the ubiquitinated EGFR. This is an important study, which describes a novel mechanism by which LAPTM4B inhibits EGFR sorting, possibly explaining the role of LAPTM4B as a tumorigenic protein upregulated in various cancers.

The strength of this study is its solid biochemical data. However, its weakness is the microscopy analysis in which many important conclusions are based on heavily processed and difficult to interpret images. In particular, most micrographs showing co-localization of various molecules
appear to be overinterpreted (most critical of them listed below). Moreover, some of the findings seem to be inconsistent with the authors' general conclusions.

There is also a need for clarification of some methods used for quantification and statistical analysis of experiments throughout the manuscript (most significant ones listed below).

Major points:

1. Figure 1 E and F panels are of poor quality and show only marginal co-localization of LAPTM4B with either LAMP1 or the PIP5Kιγι5 in F. These panels clearly do not support the authors' statement that "LAPTM4B is initially sorted to the limiting membrane of MVEs and then partially sorted onto ILVs as the MVE matures". This statement may be supported by the EM pictures, but without some sort of additional markers it is difficult to judge whether the two panels indeed represent late or early MVEs. The number of internal vesicles may not be enough to make this distinction.

2. The localization of the endosomal markers to one side of the nucleus is very peculiar. Endosomes usually populate the whole cell except the recycling compartment, which is pericentrosomal.

3. PIP5Kιγι5 was previously shown by the authors to interact with sorting nexin 5 (SNX5) and regulate Hrs binding to EGFR and sorting into intraluminal vesicles during MVB maturation. Given that both LAPTM4B and SNX5 bind PIP5Kιγι5 and PtdIns(4,5)P2 but exert opposing effects on EGFR sorting and degradation, how does the availability of LAPTM4B for PIP5Kιγι5 affect SNX5 function? Do the two proteins compete for PIP5Kιγι5, such that overexpression of LAPTM4B and its knockdown affect SNX5 binding to PIP5Kιγι5? While this issue was briefly mentioned in the Discussion, this Reviewer finds it important to address this issue experimentally (under the LAPTM4B or SNX5 overexpression and/or knockdown conditions).

4. The promiscuity of lipid binding by the N-terminus of LAPTM4B does not show a particularly strong PI(4,5)P2 preference, in fact mono-PIs are stronger binders and the PI3P binding may suggest that PI3P would occupy this site in the cells. In light of this finding it is really curious how the PI(4,5)P2 specificity is achieved at the level of PIP5Kιγι5-CT interaction.

5. Fig. 2E shows enhanced rate of disappearance of EGF-Alexa555 fluorescence in LAPTM4B-depleted cells. This is interpreted by the authors as a result of enhanced degradation of EGFR that complements their biochemical data. However, in a similar experiment, a higher concentration of EGF-Alexa555 was added to cells preincubated with EGF. Here, LAPTM4B depleted cells have less EGF-Alexa555 signal (same as in 2F), but in this case the authors interpret this as an EGFR recycling defect (based on the fact that cells were pre-incubated with EGF). It is necessary to support these conclusions with the use of proper markers of recycling endosomes and/or recycling cargos such as transferrin. Furthermore, the authors show and quantify "recycled EGFR" although these samples only show EGF (without staining against the receptor). Why we never see any EGFR staining in the plasma membrane? The recycling experiments are really confusing in the way they are presented.

6. The arguments built around data shown in Fig. 3 are quite unconvincing. There is an increased co-localization of EGFR with EEA1 after LAPTM4B knock-down and a decrease in co-localization with LAMP1. The authors argue that this is due to an accelerated degradation. Why should enhanced degradation increase the retention of the receptor in the EEA1 compartment? In control si cells there is no effect of chloroquine treatment on EEA1 retention of the receptor (120 min), so lysosomal degradation does not seem to have an impact on EGFR trafficking through the early endosomal compartment.

7. How was the EGFR co-localization with EEA1 and LAMP1 in Fig. 3A-D quantified? How did the authors arrive to the number "% EGFR colocalized with EEA1"? There is no mention of the colocalization analysis used and what does this scale represent. This is especially relevant as none of the picture show a full cell only a subset next to the nucleus.

8. Does the quantification shown in Fig. 3F represent analysis of many cells from a single experiment?

9. The effects of chloroquine on EGFR degradation should be also demonstrated biochemically.

10. In Fig. 4C and D how did the authors distinguish early and late MVEs for their quantification? Is it again based on the number of internal vesicles?

11. In experiments with constitutively active Rab5-what was the criteria used to determine the "% luminal EGF"?

12. It is strange that Hrs shows almost perfect co-localization with LAPTM4B (Fig. 6E) when it shows no co-localization with EEA1. Hrs and EEA1 are known to be in largely overlapping compartments. How can this apparent discrepancy be explained?

13. Figure 6l does not show what the authors describe in the text: wild-type L4B does not have an effect on EGFR degradation contrary to what the authors state. Also, these effects are smaller and in

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the case of pAKT are questionable. These experiments are supposed to be mimicking more "physiological" overexpressions, yet the effects are marginal at best.

14. Similarly, the authors state that there is no effect of L4B overexpression on PAR1 degradation (Fig. S5E and F). The Figure shows otherwise.

15. I am not sure the model accounts for all the findings. For example, how would the 6RQ mutant be more potent in its biological effects when it cannot interact with the lipid and cannot recruit the PIP5K? Would the PIP5K recruitment induce a positive feed-back loop that does increase recruitment, more PIP2, more recruitment etc? What would break this cycle?

Minor points:
1. LAPTM4B localization is interchangeably referred to as lysosomal, endosomal and late-endosomal throughout the manuscript.
2. Nedd4 should be added to the model shown in Fig. 7 as it was shown in this study to play an integral role in LAPTM4B regulation of Hrs ubiquitination and EGFR degradation.

Response to the Referees' Comments

Referee #1:
This manuscript describes a novel function for the cancer biomarker LAPTM4B as negative regulator of endosomal sorting of EGFRs. The authors identified LAPTM4B as an interactor of the endosomal PIP kinase PIPKgi5. They found that its overexpression inhibited EGF-induced endosomal sorting and lysosomal degradation of EGFRs whereas its depletion had the opposite effects. Mechanistically, it was found that LAPTM4B promotes ubiquitination of the ESCRT-0 subunit Hrs, a condition previously reported to attenuate the activity of Hrs, apparently by promoting interaction between Hrs and the E3 ubiquitin ligase Nedd4. The authors also found that the basic N-terminus of LAPTM4B interacts with various phospholipids and that this was abolished by mutation of 6 arginine residues to glutamine. Interestingly, the 6RQ mutant was found to inhibit EGFR degradation even stronger than wild-type LAPTM4B, and the authors suggest that PI(4,5)P2, generated by PIPKgi5, serves as negative regulator of LAPTM4B.

Overall this study contains excellent imaging and biochemical data, which are well supported by quantifications. Given the interest for LAPTM4B and EGFR in cancer, the present story should be of significant interest.

We thank the reviewer for the supportive comments and have addressed the specific comments below.

Major point:

The idea that LAPTM4B binds PI(4,5)P2, and that this is of functional importance, is not well supported by the lipid blot in Fig. 5F. Here, the strongest binding of LAPTM4B-N is to PE, PI3P and P15P whereas binding to PI(4,5)P2 is barely detectable. The ability of PI(4,5)P2 to stimulate the LAPTM4B-PIPKgi5 interaction could therefore be different from the one proposed. Because PI(4,5)P2 binding is central in the proposed model, the authors should investigate further the binding between LAPTM4B and phospholipids using alternative assays.

We have now added liposome-binding assay to test phosphoinositide binding of the LAPTM4BN-terminus (L4B-N). Consistent with the PIP strips data (Figure 5F), L4B-N also binds to multiple phosphoinositides including PtdIns(4,5)P2 in liposome-binding assay (Figure 5J). It is important to note that in vitro binding affinity does not necessarily correlate with functional relevance. For example, sorting nexin 5 (SNX5) binds all the phosphoinositides including PI3P with higher affinity than with PtdIns(4,5)P2 in liposome-binding assay, but both PI3P and PtdIns(4,5)P2 equally promote the SNX5 interaction with Hrs in vitro (Sun et al, 2013). Another example is the IQGAP1-C-terminus that binds most of other phosphoinositides with much higher affinity than with PtdIns(4,5)P2, but only PtdIns(4,5)P2 specifically inhibited the intramolecular interaction within the IQGAP1-C-terminus (Choi et al, 2013), likely due to a specific conformational change induced by PtdIns(4,5)P2 binding. Both SNX5 and IQGAP1 interact with
PIP 5-kinases that generate PtdIns(4,5)P2 that would be spatially generated in close proximity. Thus, it is not surprising that the L4B-N interaction with PIPKIγ5-C tail is specifically stimulated by PtdIns(4,5)P2, as PIPKIγ5 generates PtdIns(4,5)P2. It is also important to note that the Nterminus in the full length LAMPT4B is tethered to the membrane interface and this may not only change the specificity of the interaction with phosphoinositides but would constrain and orient the association of the PtdIns(4,5)P2 generated by the associated PIPKIγ5 (see Figure 7F).

**Minor point:**

Since Nedd4 is thought to bind directly to Hrs, it is not evident how LAPTM4B would enhance this interaction. Any further insight on this would be helpful.

This is an important point that we have now discussed in the revised manuscript. It is likely that without LAPTM4B the direct interaction between Nedd4 and Hrs is weak, and LAPTM4B may interacts with both of them changing their structures so that they bind with higher affinity. Or alternatively, LAPTM4B may function as a scaffold to link Nedd4 and Hrs, as LAPTM4B has two PY motifs (L/PPXY) while Hrs has only one (Figure R1). See also comment 2 from Reviewer 2.

![Figure R1](image_url)

**Figure R1.** Human Hrs sequence (GenBank: BAA23366.1) with the PY motif highlighted.

**Referee #2:**

In this work the impact of LAPTM4B on EGFR endosomal sorting and degradation is investigated. A number of solid and straightforward experiments are conducted that cogently demonstrate that LAPTM4B is able to inhibit EGFR sorting into intraluminal vesicles (ILVs) of late endosomes/MVBs. Importantly, this function is inhibited by binding of LAPTM4B to PtdIns(4,5)P2 and PIPKIγ5, unveiling a fine-tuned regulation of the process. Interestingly, within cells population the authors were able to correlate high levels of LAPTM4B to the inhibition of EGFR sorting into ILVs in normal growing conditions, in absence of perturbations. Molecularly, the authors showed that LAPTM4B could facilitate Nedd4-Hrs binding, thus increasing Hrs ubiquitination. As a consequence, they suggest that Hrs is inhibited (via an intramolecular interaction between Ub and ubiquitin-interacting motif within the Hrs moiety), thus not any more able to recruit the EGFR for sorting and degradation into the late endosomes/MVBs.

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We thank this reviewer for the positive comments.

There are two points that need to be strengthened:

1) One critical issue is what is the fate of the EGFR that is not targeted to the intraluminal vesicles of MVBs due to inhibition by LAPTM4B. Authors measured EGFR recycling in a very indirect and not quantitative way, which actually cannot discriminate between alterations in recycling from differences in internalization rates. Authors need to provide a more quantitative and reliable assay to measure EGFR recycling, e.g. based on radioactive EGF ligand and/or

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FACS-based methods using EGFR antibodies to follow recycling of the receptor (see, for instance, Sorkin and Duex 2010; Sigismund et al., 2008).

This is an important point. We now have performed FACS-based EGFR recycling assay in control and LAPTM4B knockdown cells. The data reveal that EGFR recycling was not affected upon loss of LAPTM4B (Figure S3), indicating that LAPTM4B inhibition of EGFR intraluminal sorting does not promote EGFR recycling, consistent with prolonged EGFR retention at LAPTM4B positive endosomes in LAPTM4B expressing cells (Figures 3E and 3F). These combined data support that the LAPTM4B-promoted EGFR signaling comes from endosomes instead of the plasma membrane. In fact, endosomes are emerging as an essential site for receptor tyrosine kinase signaling (Murphy et al., 2009).

2) A second issue concerns the mechanism of action of LAPTM4B on Nedd4 and Hrs ubiquitination. First, authors should provide more controls for the Hrs ubiquitination assay. Indeed, authors overexpress His-Ubiquitin but they did not show any western blot (WB) to control for equal level of transfection in the different samples. This control is mandatory. In addition, authors should confirm ubiquitination of Hrs in the opposite way, by immunoprecipitating Myc-Hrs and blotting anti-Ub (anti-His might be very dirty in WB, while for Ub different commercially available antibodies exist that work well in WB, e.g. P4D1 from Santa Cruz or FK2 from BIOMOL). Finally, in previous reports it was shown that LAPTM4B possesses PY motifs through which it can bind to Nedd4 (Milkerite and Rotin, 2011). This mechanism is not contemplated by the authors. However, it would be important to show that LAPTM4B mutant in PY motifs abolishes its binding to Nedd4 and, as a consequence, revert LAPTM4B phenotypes (i.e. increased Hrs ubiquitination and decreased EGFR degradation). This experiment would provide a final prove that LAPTM4B action on EGFR is via Hrs ubiquitination. Since Hrs has no PY motifs, one intriguing possibility -that can be discussed - might be that LAPTM4B serves as an adaptor/scaffold between Hrs and Nedd4, as shown for ARTs adaptors in Yeast and, more recently, for ARRDC3 in mammals (Nabhan et al., 2010).

These are important and constructive comments. We have now added blots for HA-Ub levels in the whole cell lysates (Figures 6B and 6C). We believe assaying Hrs ubiquitination by immunoprecipitating (IP) Hrs has its intrinsic difficulties, because other ubiquitinated proteins would be co-IP’ed with Hrs interfering with the detection of Hrs ubiquitination, and denatured IP would on the other hand compromise the IP efficiency. Additionally, while by purifying ubiquitinated proteins the mono-ubiquitinated Hrs (mono-Ub-Hrs) was clearly detected as the major band of Ub-Hrs (Figures 6B and 6C), by IP Hrs and blotting with anti-Ubiquitin (or anti-HA) the band corresponding to mono-Ub-Hrs was not detectable, possibly because the mono-Ub-Hrs was not IP’ed by the antibody.

We have generated the PY motif mutant for LAPTM4B (P296/312A, or 2PA) and assessed the effects of this mutant on EGFR degradation and signaling. As shown in Figure 6I, LAPTM4B-2PA lost Nedd4 interaction, but showed normal Hrs interaction, with enhanced interaction with PIPKIγ5. In support of a requirement for the LAPTM4B-Nedd4 interaction in LAPTM4B function, we observed inhibition of EGFR degradatin in LAPTM4B-WT overexpressing cells but no inhibition in LAPTM4B-2PA mutant overexpressing cells (Figures 6J and 6K).

Minor points:

Figure 2F. It is not clear what is required for. Authors compared two doses after different pulse and chase times. What are the conclusions? I would remove this panel (or put in the supplementary) since it does not add any valuable information.

This panel shows how much of total EGFR is internalized to endosomes and tracked for degradation in Figure 2E. We compared the two EGFR stimulation conditions to get a sense of what percentage of EGFR is internalized after a 3 min pulse of 25 ng/ml Alexa-555-EGF in Figure 2E, assuming that continuous stimulation with 100 ng/ml EGF for 15 min induces accumulation of most EGFR at endosomes. The result indicates that under the condition used in Figure 2E, only around 10% of receptor is internalized. Thus, the data in Figures 2E and 2G represent the degradation of only a very small pool of EGF stimulated EGFR. We have clarified
our interpretations of these experiments in the revised manuscript.

Supplementary Figure S5 C-F. Authors try to show that LAPTM4B-dependent mechanism is specific for EGFR, while not applying to other receptors. However, data do not support this conclusion. In addition authors cite literature in support of the idea that degradation of c-Met does not require Hrs. However there are reports show the opposite (e.g. Hammond et al., 2003; Row et al., 2005; Abella et al., 2005). I strongly suggest to remove this part, which in my opinion does not add anything to the final message of the paper.

We thank the reviewer for bringing these papers into our attention. We noticed that the initial work by Hammond et al., 2003 reported a “modest retardation” of c-Met degradation in Hrs knockout cells, and the other two papers in 2005 only suggested an involvement of Hrs in c-Met signaling. However, we did not observe a change of c-Met degradation upon knockdown of Hrs or PIPKIγ5, or upon LAPTM4B overexpression in MDA-MB-231 cells. We think both our data and others’ are consistent and we would like to retain these data in the supplementary figures, but we have changed the wording from “not required” into “not essential”, the latter of which we believe is more appropriate.

The PAR1 data is an important control. First, though EGFR degradation is blocked upon LAPTM4B overexpression, PAR1 degradation is actually accelerated in LAPTM4B overexpressing cells, which rules out the possibility that LAPTM4B overexpression causes a general dysfunction of the lysosome or blocks the broad degradation of receptors in the lysosome. Second, it is known that PAR1 degradation does not require PAR1 ubiquitination or Hrs binding. In fact, PAR1 directly associates with an ESCRT-3 subunit for lysosomal targeting (Dores et al, 2012; Gullapalli et al, 2006). Thus, these data provide an additional control that LAPTM4B inhibits EGFR degradation by inhibiting Hrs but not downstream ESCRT subunits.

Referee #3:

In this manuscript Tan et al. describe a novel function to the lysosomal protein, LAMTM4B, namely, the regulation of intraluminal sorting of the activated EGF receptor (EGFR). The authors show that LAMTM4B overexpression delays the degradation of EGF receptors and prolongs its signaling activity. Conversely, LAMTM4B depletion accelerated the termination of EGF signaling and EGFR degradation. The authors also demonstrated a direct interaction between PIPKIγ isoform 5 and LAMTM4B N-terminus that appears to be regulated by PI(4,5)P2. They showed that the membrane-adjacent polybasic domain of the LAMTM4B at the N-terminus associates with endosomal PtdIns(4,5)P2, which stabilizes the PIPKIγ5-LAMTM4B interaction. To explain the mechanism of the LAMTM4B effect on EGF sorting, the authors showed that LAMTM4B acts as a negative regulator of Hrs-driven EGFR intraluminal sorting at the MVB. This is mediated by the interaction of LAMTM4B with Nedd4 ubiquitin ligase with enhanced ubiquitination of Hrs, thus inhibiting its interaction with the ubiquitinated EGFR. This is an important study, which describes a novel mechanism by which LAPTM4B inhibits EGFR sorting, possibly explaining the role of LAPTM4B as a tumorigenic protein upregulated in various cancers. The strength of this study is its solid biochemical data. However, its weakness is the microscopy analysis in which many important conclusions are based on heavily processed and difficult to interpret images. In particular, most micrographs showing colocalization of various molecules appear to be overinterpreted (most critical of them listed below). Moreover, some of the findings seem to be inconsistent with the authors’ general conclusions. There is also a need for clarification of some methods used for quantification and statistical analysis of experiments throughout the manuscript (most significant ones listed below).

We thank the reviewer for recognizing the importance and strength of this study. We have addressed the specific comments below with additional experiments and editing clarifications.

Major points:

1. Figure 1 E and F panels are of poor quality and show only marginal co-localization of LAPTM4B with either LAMP1 or the PIP5KIγ5 in F. These panels clearly do not support the authors’ statement that "LAPTM4B is initially sorted to the limiting membrane of MVEs and then partially sorted onto ILVs as the MVE matures". This statement may be supported by the EM
pictures, but without some sort of additional markers it is difficult to judge whether the two panels indeed represent late or early MVEs. The number of internal vesicles may not be enough to make this distinction.

The comments regarding LAPTM4B localizations are important (see also comment 12). We have put much effort into re-examining the localization of endogenous LAPTM4B and have quantified the colocalization. This reveals that ~45% of LAPTM4B is colocalized with EEA1 and ~60% with LAMP1. On the other hand, ~60% of either EEA1 or LAMP1 is colocalized with LAPTM4B (Figures 1E and 1F). These data indicate that although less LAPTM4B is colocalized with EEA1, the majority of either EEA1 or LAMP1 compartments are LAPTM4B positive. This is consistent with partial LAPTM4B colocalization with PIPKIγ5 and Hrs.

We agree that the immunofluorescence (IF) data alone do not support the sentence pointed out by the reviewer due to resolution limit of the IF method. However, the IF data provided important controls for the EM, as they clearly showed LAPTM4B localization to both EEA1 and LAMP1 compartments. It is also important to note that multivesicular endosomes (MVEs) are defined on the morphological observation of small intraluminal vesicles (ILVs) (50-80 nm diameter) and the morphological differences between early and late MVEs are the number of ILVs. Thus, we believe additional markers for EM are not necessary.

2. The localization of the endosomal markers to one side of the nucleus is very peculiar. Endosomes usually populate the whole cell except the recycling compartment, which is pericentrosomal.

In MDA-MB-231 cells the endosomes are always well polarized on one side of the nucleus (see Figure R2).

3. PIP5KIγ5 was previously shown by the authors to interact with sorting nexin 5 (SNX5) and regulate Hrs binding to EGFR and sorting into intraluminal vesicles during MVB maturation. Given that both LAPTM4B and SNX5 bind PIP5KIγ5 and PtdIns(4,5)P2 but exert opposing effects on EGFR sorting and degradation, how does the availability of LAPTM4B for PIP5KIγ5 affect SNX5 function? Do the two proteins compete for PIP5KIγ5, such that overexpression of LAPTM4B and its knockdown affect SNX5 binding to PIP5KIγ5? While this issue was briefly mentioned in the Discussion, this Reviewer finds it important to address this issue experimentally (under the LAPTM4B or SNX5 overexpression and/or knockdown conditions)

We have explored the interaction of PIP5KIγ5 with LAPTM4B and SNX5. To narrow down the interaction regions for LAPTM4B and SNX5 on the C-tail of PIP5KIγ5, a series of C-terminal deletion mutants of PIP5KIγ5 were generated and used in co-immunoprecipitation experiments to characterize interactions with LAPTM4B or SNX5. The data indicate that LAPTM4B interaction required the very C-terminal part of the PIP5KIγ5 C-tail, while SNX5 interaction required the N-terminal part of the C-tail (Figures 7A-C), suggesting that LAPTM4B and SNX5 may not
compete for PIPKIγi5 interaction. In fact, overexpression of PIPKIγi5 promoted the interaction between LAPTM4B and SNX5 (Figure 7D). We previously showed that SNX5 inhibits Hrs ubiquitination by excluding Nedd4 recruitment to Hrs (Sun et al, 2013). As LAPTM4B facilitates the Hrs-Nedd4 association, it is likely that SNX5 interacts with LAPTM4B to inhibit LAPTM4B function. To test this, we examined if SNX5 inhibits LAPTM4B interaction with Nedd4 or Hrs. And we found that SNX5 overexpression strongly suppressed LAPTM4B interaction with Hrs (Figure 7E) but not Nedd4 (Figure S6A). Our combined data support that PIPKIγi5 inhibits the function of LAPTM4B by both generating PtdIns(4,5)P2 and recruiting SNX5 (Figure 7F).

4. The promiscuity of lipid binding by the N-terminus of LAPTM4B does not show a particularly strong PI(4,5)P2 preference, in fact mono-Pi5s are stronger binders and the PI3P binding may suggest that PI3P would occupy this site in the cells. In light of this finding it is really curious how the PI(4,5)P2 specificity is achieved at the level of PIP5Kγi5-CT interaction.

See also discussion above for Reviewer 1. We have performed liposome-binding assay to test lipid binding of the LAPTM4B N-terminus. This reveals that, similar to PIP strips assay (Figure 5F), LAPTM4B N-terminus also binds multiple phosphoinositides including PtdIns(4,5)P2 in liposome binding assay (Figure 5J). Binding affinity does not always correlate with functional relevance. SNX5 and IQGAP1 are another two examples that are functionally regulated by PtdIns(4,5)P2 but bind all phosphoinositides in liposome binding assays (Choi et al, 2013; Sun et al, 2013). In addition, as PtdIns(4,5)P2 is the product of PIPKIγi5 (Schill & Anderson, 2009), this also explains the specificity of PIPKIγi5 interaction.

5. Fig. 2E shows enhanced rate of disappearance of EGF-Alexa555 fluorescence in LAPTM4B depleted cells. This is interpreted by the authors as a result of enhanced degradation of EGFR that complements their biochemical data. However, in a similar experiment, a higher concentration of EGF-Alexa555 was added to cells preincubated with EGF. Here, LAPTM4B depleted cells have less EGF-Alexa555 signal (same as in 2F), but in this case the authors interpret this as an EGFR recycling defect (based on the fact that cells were pre-incubated with EGF). It is necessary to support these conclusions with the use of proper markers of recycling endosomes and/or recycling cargos such as transferrin. Furthermore, the authors show and quantify "recycled EGFR" although these samples only show EGF (without staining against the receptor). Why we never see any EGFR staining in the plasma membrane? The recycling experiments are really confusing in the way they are presented.

The EGF-Alexa555 degradation assay monitored EGF signal after 1-3 h. In the original EGFR recycling assay cells were pre-incubated with EGF to internalize EGFR and the new synthesis of EGFR was inhibited by cycloheximide. After allowing for recycling for 1 h, the binding of EGF-Alexa555 (15 min treatment) to the cells reflects the amount of EGFR recycling (Raiborg et al, 2008). However, the original quantification method has its limitation, and we have re-examined EGFR recycling more quantitatively by FACS, as raised by reviewer 2. The new data indicate that loss of LAPTM4B does not affect EGFR Recycling rate (Figure S3), supporting that LAPTM4B enhances endosomal EGFR signaling, consistent with EGFR stabilization at LAPTM4B positive endosomes (Figure 3E).

6. The arguments built around data shown in Fig. 3 are quite unconvincing. There is an increased co-localization of EGFR with EEA1 after LAPTM4B knock-down and a decrease in colocalization with LAMP1. The authors argue that this is due to an accelerated degradation. Why should enhanced degradation increase the retention of the receptor in the EEA1 compartment? In control si cells there is no effect of chloroquine treatment on EEA1 retention of the receptor (120 min), so lysosomal degradation does not seem to have an impact on EGFR trafficking through the early endosomal compartment.

We realized that the LAPTM4B knockdown phenotype here is quite unique, as most previous studies showed enhanced EGFR-EEA1 colocalization after a block of EGFR at early endosomes. However, it is important to note that the increased colocalization of EGFR with EEA1 does not always mean enhanced EGFR retention in early endosomes; in the case of LAPTM4B knockdown, it in fact reflects less EGFR in LAMP1 compartment due to accelerated
lysosomal delivery and subsequent degradation. At 120 min, in control cells, only 37% of EGFR is degraded, but in siLAPTM4B cells, ~75% of EGFR is lost (Figure 2). This may explain why chloroquine treatment had a more evident effect on the EGFR-EEA1 colocalization in LAPTM4B-knockdown cells than in control cells.

7. How was the EGFR co-localization with EEA1 and LAMP1 in Fig. 3A-D quantified? How did the authors arrive to the number "% EGFR colocalized with EEA1”? There is no mention of the co-localization analysis used and what does this scale represent. This is especially relevant as none of the picture show a full cell only a subset next to the nucleus.

We have now clarified the methods for colocalization analysis in the revised manuscript. Images were background subtracted and splitted into individual channels (eg. channel 1 for EGFR, channel 2 for EEA1), and the colocalization quantification of signals from two individual channels was performed using the Coloc 2 plugin of Fiji (ImageJ). The thresholded Manders M1 coefficient was expressed as percentages (eg. M1 = 0.3 was expressed as 30%) to show the fraction of intensities in channel 1 above threshold that is colocalized with intensities in channel 2 above threshold.

We did not intentionally show only a subset of a cell instead of a full cell in Figures 3A and 3C. Because the EGFR (and EEA1/LAMP1) signals are highly concentrated at endosomes in all of these conditions, we could not see additional signals outside of the endosome-rich region, as exemplified in Figure 3E. For images of each condition in Figures 3A and 3C, we have shown all the visible signals of one representative cell. Showing more background region (purely black) does not add any further information.

8. Does the quantification shown in Fig. 3F represent analysis of many cells from a single experiment?

Yes, this is one representative analysis out of three independent experiments. We have now clarified this in the figure legends.

9. The effects of chloroquine on EGFR degradation should be also demonstrated biochemically. We initially had a control that chloroquine blocks EGFR degradation in A431 cells (Figure S2B, bottom). We have now added the same control for MDA-MB-231 cells in Figure S2D.

10. In Fig. 4C and D how did the authors distinguish early and late MVEs for their quantification? Is it again based on the number of internal vesicles?

The quantification is based on all MVEs since LAPTM4B is targeted to both early and late MVEs. All endosomes with gold particles and internal vesicles are included.

11. In experiments with constitutively active Rab5-what was the criteria used to determine the "% luminal EGF"?

Quantification of EGF on the limiting membrane and within the endosomal lumen was done as described (Trajkovic et al., 2008). Central images of endosomes (diameter > 2 µm) were taken with the GFP-Rab5Q79L outline as a reference. For quantification, the GFP-Rab5Q79L outline was also used as a reference to determine the EGF localization on the limiting membrane. EGF localized inside the GFP-Rab5Q79L outline was considered as intraluminal EGF. The total intensities of endosomal EGF fluorescence and the intensities inside the GFP-Rab5Q79L outline were quantified in ImageJ.

12. It is strange that Hrs shows almost perfect co-localization with LAPTM4B (Fig. 6E) when it shows no co-localization with EEA1. Hrs and EEA1 are known to be in largely overlapping compartments. How can this apparent discrepancy be explained?

This is a very important point that we have now fully addressed. Clearly, Hrs and EEA1 are largely overlapping, which has also been confirmed on our hands. The observation that most Hrs also co-localizes with LAPTM4B encouraged us to re-consider the localization of LAPTM4B. We have now re-examined and quantified LAPTM4B colocalization with EEA1 and
LAMP1 (Figures 1E and 1F). Both our previous and current data indicate that while only part of LAPTM4B colocalizes with EEA1, most EEA1 endosomes are positive for LAPTM4B, indicating that LAPTM4B targets to both early and late endosomes. This is consistent with partial LAPTM4B colocalization with PIPKIγ5 and Hrs. See also Comment 1.

13. Figure 6I does not show what the authors describe in the text: wild-type LAB does not have an effect on EGFR degradation contrary to what the authors state. Also, these effects are smaller and in the case of pAKT are questionable. These experiments are supposed to be mimicking more "physiological" overexpressions, yet the effects are marginal at best.

The effects of LAPTM4B overexpression on EGFR degradation and signaling were shown in Figures 2H-K. However, robust overexpression of either WT or 6RQ LAPTM4B had too strong effects on EGFR degradation, which totally masked the differences between WT and the mutant. Thus, we compared two pools of cells that overexpress more physiological levels of LAPTM4B-WT or -6RQ for their effects on EGFR degradation and signaling. For this, single clones were selected and the expression levels of ectopic LAPMT4B were examined by staining with anti-Flag. Low expression clones were selected and combined into a polyclonal pool for either LAPTM4B-WT or 6RQ. We have now clarified this in the updated figure legends.

14. Similarly, the authors state that there is no effect of L4B overexpression on PAR1 degradation (Fig. 5E and F). The Figure shows otherwise.

We clarified it as “did not inhibit” but not that there was “no effect”. In fact, LAPTM4B overexpression accelerated PAR1 degradation. We have now described this result more clearly in the revised manuscript.

15. I am not sure the model accounts for all the findings. For example, how would the 6RQ mutant be more potent in its biological effects when it cannot interact with the lipid and cannot recruit the PIP5K? Would the PIP5K recruitment induce a positive feed-back loop that does increase recruitment, more PIP2, more recruitment etc? What would break this cycle?

The current study and our previously work indicate that PIPKIγ5 and PIP2 are negative regulators of LAPTM4B. In this scenario, the 6RQ mutant, lacking PIPKIγ5 and PIP2 binding, showed enhanced activity in promoting Hrs-Nedd4 association, increasing Hrs ubiquitination and inhibiting EGFR degradation. We have now improved the description of our model with additional clarifications, including the explanation of Nedd4 in this pathway (as suggested by this reviewer in Minor point 2).

We appreciate the comment regarding the positive feedback loop for PIPKIγ5 recruitment to LAPTM4B. However, we do not have a clear answer to this question now. Both PIPKIγ5 and LAPTM4B are posttranslational modified. Possibly, upstream signals may lead to posttranslational modifications of PIPKIγ5 and/or LAPTM4B that would accelerate or block this positive feedback loop. This is one of our future directions that are being explored for another manuscript.

Minor points:

1. LAPTM4B localization is interchangeably referred to as lysosomal, endosomal and late endosomal throughout the manuscript.

Based on the quantification of LAPTM4B localization (see comment 1), we have now consistently used “endosomal” throughout the manuscript.

2. Nedd4 should be added to the model shown in Fig. 7 as it was shown in this study to play an integral role in LAPTM4B regulation of Hrs ubiquitination and EGFR degradation.

We have now added this.

References:

Choi S, Thapa N, Hedman AC, Li Z, Sacks DB, Anderson RA (2013) IQGAP1 is a novel...
phosphatidylinositol 4,5 bisphosphate effector in regulation of directional cell migration. 

The EMBO Journal 32: 2617-2630


Thank you for submitting the revised version of your manuscript entitled 'LAPTM4B is a PtdIns(4,5)P2 Effector that Regulates EGFR Signaling, Lysosomal Sorting and Degradation'. I have now received the reports from all referees and I am happy to inform you that they support publication pending minor amendments to the manuscript.

As you will see below, referee #3 has two remaining concerns that I would like to ask you to address:

1) Referring to a luminal localization of LAMPTM4B in lysosomes should be restricted to Figure 1I.

2) To address the second point of this referee regarding figure 3, I suggest that you amend the text of your manuscript and/or figure legend to better explain and outline what the observed data suggest. For this, it might be good to explain how the analysis was performed for Figures 3B and D - I assume that you analyzed all EGFR punctae present under each condition (= 100%) for co-localization with EEA1/Lamp1 (= depicted %). Maybe you could mention at this point again that the total EGFR levels (and punctae?) are reduced upon LAPTM4B knock-down.

I am therefore formally returning the manuscript to you for a final round of minor revision. Once we have received the final version, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

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REFEREE COMMENTS

Referee #1:

The authors have successfully addressed the concerns I had. In my opinion, this revised manuscript is well suited for publication in EMBO Journal.
Referee #2:

The authors have addressed all major concerns I have raised in the first round of revision. As a minor point, I still think that data in supplementary Figure S6 on Met and Par1 are not totally convincing and I suggest to remove them. Nonetheless, I believe that the work is straightforward and the molecular mechanism is deeply dissected, helping to clarify how EGFR fate and signaling are regulated at the sorting station by LAPTM4B/Nedd4/Hrs interplay. Importantly, this may represent also the basis to rationalize LAPTM4B overexpression in cancer. For this reasons I suggest publication in EMBO J.

Referee #3:

The authors have addressed most of my critical comments and provided extra explanations and clarifications. I still found a few issues that were not properly addressed in my opinion. However, these may not reach a level of dissatisfaction that would warrant holding the paper back.

-P7, Luminal localization of LAPTM4B cannot be judged in panels E and H, so this statement should be referred to Fig.1I.

-This reviewer still does not understand the explanation of the results shown in Fig. 3B and D and the authors response to this comment did not make it more understandable.

2nd Revision - authors’ response 11 December 2014

Referee #1:

The authors have successfully addressed the concerns I had. In my opinion, this revised manuscript is well suited for publication in EMBO Journal.

We thank this reviewer for support of publication.

Referee #2:

The authors have addressed all major concerns I have raised in the first round of revision. As a minor point, I still think that data in supplementary Figure S6 on Met and Par1 are not totally convincing and I suggest to remove them. Nonetheless, I believe that the work is straightforward and the molecular mechanism is deeply dissected, helping to clarify how EGFR fate and signaling are regulated at the sorting station by LAPTM4B/Nedd4/Hrs interplay. Importantly, this may represent also the basis to rationalize LAPTM4B overexpression in cancer. For this reasons I suggest publication in EMBO J.

We thank this reviewer for support of publication. We have agreed with all of this reviewer’s excellent comments and suggestions, except that we respectfully disagree with the reviewer on the removal of the data in Figure S6. We have replicated this data many times and clearly LAPTM4B or the PIPKιγi5 pathway do not regulate sorting of these two receptors. This shows a degree of receptor specificity and that the endosomal pathway is not disrupted generally and is important to be presented here.

Referee #3:

The authors have addressed most of my critical comments and provided extra explanations and clarifications. I still found a few issues that were not properly addressed in my opinion. However, these may not reach a level of dissatisfaction that would warrant holding the paper back.

We thank this reviewer for support of publication.
-P7, Luminal localization of LAPTM4B cannot be judged in panels E and H, so this statement should be referred to Fig.1I.

We have now only referenced the luminal localization of LAPTM4B to Fig. 1I.

-This reviewer still does not understand the explanation of the results shown in Fig. 3B and D and the authors response to this comment did not make it more understandable.

We have added more details to the figure legends and result description to improve our explanations of these data. Details regarding co-localization quantification are included in the Materials and Methods section of the main text.