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Transport through recycling endosomes requires EHD1 recruitment by a phosphatidylserine translocase

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1st Editorial Decision 26 August 2014

Thank you for submitting your manuscript entitled 'Phosphatidylserine flipping is essential for the recruitment of the membrane fission protein EHD1'. I have now received reports from three referees, which are enclosed below.

As you will see, all referees appreciate your findings. However, they raise various concerns that need to be addressed. I will not restate the concerns here, as all reports are very concise and constructive.
Given the 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 questions regarding your revision.

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

REFEREE REPORTS

Referee #1:

The manuscript by Lee and colleagues provides compelling evidence that the phospholipid flippase activity of ATP8A1 is required for transport of proteins through recycling endosomes in mammalian

cells. They also show that EHD1, an ATPase potentially involved in driving fission of tubular transport intermediates, requires ATP8A1 activity and enrichment of PS in the cytosolic leaflet for recruitment to the RE membrane. Consistently, ATP8A1 knockdown cells display abnormal tubular recycling endosomes that suggests a fission defect. The PS translocase activity in recycling endosomes can also be provided by ATP8A2, within which mutations cause intellectual disability in humans. Furthermore, the trafficking of the transferrin receptor through recycling endosomes is disrupted in Atp8a2-/- neurons from mice. While P4-ATPases have been implicated in endosomal trafficking in yeast and C. elegans, this work provides important new information on the specific trafficking pathways requiring phospholipid flippase activity in mammalian cells and mechanistic insight into why PS translocation is required through the connection to EHD1.
This study was very nicely done and I have only one significant concern to voice (point 1 below). The data are technically sound, well controlled and support the conclusions of the work. There are a number of minor issues listed below (points 2 - 8) that the authors should also consider during revision of their manuscript.

1. The high concentration of PS (>50%) required for binding of EHD1 to liposomes is of questionable physiological significance (Fig 5). Others have reported significant binding of EHD1 to liposomes containing 10% PS, 35%PE and PI4,5P2 or PI4P (Blume et al). It seems possible (even likely) that other lipid factors are contributing to EHD1 recruitment and the authors should do a better job of pinpointing the critical differences between the published observations and their work. At the minimum, a direct comparison of the Blume et al liposomes composition, +/-PS, relative to the PS/PC liposomes used in the current work should be done. The curvature of the liposomes may also make a difference and it would be worthwhile to test liposomes of varying diameter produced by extrusion. While the authors make a good case that PS is the key substrate of ATP8A1 needed for EHD1 recruitment, the observations are correlative in nature and it is possible that PE translocation is also contributing.

2. The authors should report the isoform of ATP8A1 that was cloned and used in this study. Ding et al (JBC 2000 275(30):23378-86) reported differences in apparent substrate specificity between different splice isoforms of the bovine enzyme and so it is relevant to know which human isoform was cloned.

3. Another potential site of action for ATP8a1 is the TGN and it seemed odd that a TGN marker was not examined for co-localization with ATP8A1 in Fig 1. Perhaps this could be done as well.

4. It is surprising that 100% of the NBD-PS is getting translocated in the reconstitution experiments shown in Fig 4B and that the NBD-PE translocase activity was that high. This is significantly more efficient than published reports raising concerns that the membrane integrity was compromised and the quencher was accessing the luminal leaflet during the assay. The ATP-dead control looks good and so the appropriate control is there. I don't have a specific recommendation other than to alert the authors that this result is a little suspicious.

5. In figure 6B it appears that 2XPH labels only the RE, while Lact-C2 labels the plasma membrane and RE. Is this the case? If so, why do the authors think the PS at the plasma membrane is not being recognized by 2XPH?

6. In the Chen et al 2010 PLoS Genetics paper, the Wang group localized RME-1 (EHD1 homolog) in the tat-1 mutant. They reported a modest loss of membrane association but most of the RME-1 still localized to abnormal endosomes. In this case, defects in trafficking through the RE were not attributed to a loss of RME-1 function. It would appropriate to comment in the Discussion on this modest discrepancy between the literature and the current work.

7. What is the functional relationship between evectin-2 and EHD1? Both may be effectors of PS flip by ATP8A1. Do the authors think they are both involved in scission or is evectin-2 doing something else? Does evectin-2 interact with EHD1? Perhaps this could be commented on in the Discussion.

8. From the Discussion - "Thus, although ATP8A1 contributes to the enrichment of PS in the cytosolic leaflet of RE membranes, its knockdown does not result in the complete disappearance of PS at the cytosolic leaflet. Scramblases that mediate bidirectional transbilayer movement of

phospholipids or other P4-ATPases may participate in the exposure of PS at the cytosolic leaflet in ATP8A1-depleted cells." PS is synthesized in the cytosolic leaflet of the ER and likely moves by both vesicular and nonvesicular routes to the PM and other organelles. The nonvesicular route would necessarily deliver PS to the cytosolic leaflet and may move the majority of the PS in the cell.

9. The title could better reflect the content of the paper. For example "Transport through recycling endosomes requires EHD1 recruitment by a phosphatidylserine translocase".

Referee #2:

This manuscript, by Lee et al. addresses the role of PS and PS flippases in the function of recycling endosomes. The authors first studied the localization of ATP8A1 and suggest that it localizes selectively to recycling endosomes and regulates Tf recycling and retrograde transport of CTxB, but not EGF transport to lysosomes. Depletion of ATP8A1 led to the appearance of aberrant endosomal tubules that depend upon microtubule integrity. In addition, tubule generation appears to be dependent upon loss of ATP8A1 ATPase activity. The authors provide evidence that the RE localization of the RE regulatory protein, EHD1, depends on ATP8A1 activity, and through lipid-binding assays further suggest that PS localization to RE is the mechanism that allows recruitment of EHD1 to RE. Specific markers of PS were employed to show that ATP8A1 is required to flip PS from the luminal RE membrane to the cytoplasmic (EHD1) side. Finally, the authors demonstrate that the ATP8A1 paralog, ATP8A2, can compensate for ATP8A1 loss by flipping PS from the luminal lipid layer to the cytoplasmic layer, and this paralog also regulates Tf recycling in neurons from Atp8a2-/- mice.

The role of PS (and the ATP8A1 flippase) in regulating RE function, and specifically the recruitment of EHD1 to induce fission at RE is novel and timely. The authors have addressed the function of a poorly studied but important protein, and have intriguing data. However, this reviewer finds the manuscript rather preliminary at this time, and there are significant concerns with (the rigor of) some of the conclusions drawn as well as conceptual concerns that were not addressed in the study.

Major concerns:
1) In the first figure, the authors discuss "colocalisation" but as they have not quantified any of the data, have no means to determine to what degree this quantification occurs (or does not). In addition, the data provided for the knock-down is really not compelling- the western blot is rather dirty and both siRNA2 and 3 don't appear to show any decrease. Whilst the siRNA1 does show an increase, the loading control of tubulin is also distinctly lower, leaving this reviewer concerned as to whether knock-down is occurring at all.
2) In the same figure, whereas the Tf recycling seems to be impacted by ATP8A1 knock-down, an important control should be a reinsertion of the wild-type ATP8A1 to show that the effect is not an off-target one.
3) The authors relate extensively to the aberrant tubules formed, and even show that ATP8A1 ATPase activity loss is required for their formation. There is little clarification of the significance of these aberrant structures. How are they formed? What are they? Why are they significant? If anything, the knock-down/knock-in experiment with the ATPase-deficient mutant would be better served in the Tf recycling assay noted above.
4) I have concerns about the physiological significance of the liposome assays. 40-70% PS is very high, and PE is probably not the best control. Sedimentation or flotation assays with lower concentrations would be a lot more convincing.
5) Another major question left unanswered is how EHD1 binds to PS. Daumke et al. showed that conserved lysines in the helical region of EHD2 are required for membrane sensing and curvature. Are these the lysines that contact PS, allowing charge-based binding? Or is the EH domain (as suggested by Keiken et al.) responsible for this interaction?
6) Perhaps the most major conceptual concern is how the authors relate their study to that of Giridaran et al. The latter authors presented a model suggesting that EHD1 recruitment to RE occurs via several NPF-containing proteins, Mical and Pacsin, which reportedly bind to phosphatidic acid. It's entirely possible that PS could independently recruit EHD1, regardless of Mical and Pacsin, but it's also entirely possible that PS levels indirectly affect phosphatidic acid or recruitment of Mical and Pacsin. In any case, the authors of this manuscript have not even addressed these issues and this

model, either experimentally or conceptually

Referee #3:

This is a very nice paper. It is logically complete and technically excellent: the technical scope covers experiments in cells, on vesicles, and includes assessment of transport activities of purified proteins in reconstituted systems. However, the authors are a bit disingenuous when they claim that nothing is known about P-type ATPases and vesicular transport because quite a bit has been learned in recent years through analyses in yeast of the role of Drs2 in vesicle formation at the Golgi apparatus. ATP8A1, the subject of the present paper, is a homolog of Drs2. Nevertheless, the authors have generated a substantial body of information that advances the fields of lipid asymmetry and membrane transport.

I have only some minor comments:

It is important to show the raw data for the flippase assay using purified, reconstituted proteins. This is important as the bar chart shown in Figure 4B obscures the fact that the activity signals in these flippase assays are typically very small and can be unconvincing.

Recent reviews by Bigay and Antonny (Dev Cell 2012) and Holthuis and Menon (Nature 2014) could be cited as they discuss the role of PS on the cytoplasmic face of membranes of the late secretory pathway. The latter review also discusses the regulation of P-type ATPases, something of relevance to the present paper.

The authors should comment briefly in the discussion section on the enigmatic presence of ATP8a2 in disc membranes of photoreceptor cells. What would be the role of this protein in discs given its proposed role in the secretory pathway?

1st Revision - authors' response 01 December 2014

Reviewer 1:

1. The high concentration of PS (>50%) required for binding of EHD1 to liposomes is of questionable physiological significance (Fig 5). Others have reported significant binding of EHD1 to liposomes containing 10% PS, 35%PE and PI4,5P2 or PI4P (Blume et al). It seems possible (even likely) that other lipid factors are contributing to EHD1 recruitment and the authors should do a better job of pinpointing the critical differences between the published observations and their work. At the minimum, a direct comparison of the Blume et al liposomes composition, +/-PS, relative to the PS/PC liposomes used in the current work should be done. The curvature of the liposomes may also make a difference and it would be worthwhile to test liposomes of varying diameter produced by extrusion. While the authors make a good case that PS is the key substrate of ATP8A1 needed for EHD1 recruitment, the observations are correlative in nature and it is possible that PE translocation is also contributing.
> Thank you for the valuable comments. We understand the possibility that other lipids besides PS and/or the membrane curvature may contribute to recruitment of EHD1 to the membranes. Thus, as suggested, we performed the following two experiments.
(1) As Blume et al. reported, EHD1 bound to liposomes [35% PC, 35% PE, 10% PS, 10% cholesterol, and 10% PI(4)P or PI(4,5)P2] under our experimental conditions (revised Supplementary Fig S9). We also examined the binding of EHD1 to liposomes that lack PS [45% PC, 35% PE, 0% PS, 10% cholesterol, and 10% PI(4)P or PI(4,5)P2] and found that EHD1 did not bind to these membranes (revised Supplementary Fig S9). These results indicate that PS is required for EHD1 binding to the membranes. Given that EHD1 did not bind to liposomes with a low concentration of PS in the absence of PIPs (Fig 5A and C), other lipid factors may affect the EHD1 binding to the membrane containing PS. As has been reported (Behnia and Munro, 2005; Uchida *et al.*, 2011), PIPs were not identified in the RE membrane (revised Supplementary Fig S10), which suggests that the contribution of PIPs for the EHD1 recruitment to the RE membranes

may be minimal.
(2) The effect of liposome curvature on EHD1 binding was also examined. We prepared liposomes with two different diameters by using the mini-extruder with 400 and 100 nm filters (Xu *et al.*, 2013). As shown in revised Fig 5D and E, when the curvature of liposomes increased (*i.e.*, the diameter decreased), more EHD1 bound to liposomes at the PS concentrations examined (20%, 40% and 60% PS). These results suggest that the membrane curvature influences the EHD1 binding to the membranes and that the PS concentration required for EHD1 binding to the cytosolic leaflet of RE membrane could be lowered by the curvature of RE membrane.
We've included these results and the comments in the Result section of the revised manuscript (page 13, line 11 - page 14, line 5).
We agree with the possibility that PE translocation is also contributed in the EHD1 recruitment to REs, since ATP8A1 flips PE as well as PS. In our *in vitro* experiment, 60% PE in liposomes is not sufficient for recruitment of EHD1 to the membrane (Fig 5B). We also demonstrated using PS/PE-deficient and PS-deficient CHO mutants that PS is critical for the membrane localization of EHD1 (Fig 4). However, it is possible that PE in the cytosolic leaflet may affect the PS-dependent EHD1 recruitment to the RE membranes, and we would like to elucidate this issue, for example, by producing PS-or PE-specific ATP8A1 mutant in the future study.

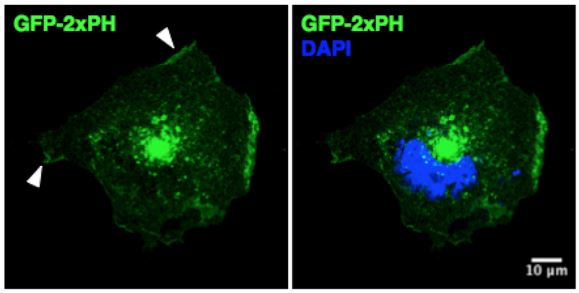
2. The authors should report the isoform of ATP8A1 that was cloned and used in this study. Ding et al (JBC 2000 275(30):23378-86) reported differences in apparent substrate specificity between different splice isoforms of the bovine enzyme and so it is relevant to know which human isoform was cloned.
> Thank you for the comment. Two isoforms of ATP8A1 are known in human according to “NCBI Gene Database”. As described in Materials and Methods, we performed PCR with cDNAs derived from HeLa cells using a set of primers that amplifies both isoforms. We obtained only one isoform (NM_001105529) which corresponds to bovine isoform β1 in the paper by Ding et al., and used it for this study. As Reviewer 1 kindly suggested, we included the gene accession number in the "Materials and Methods" (page 25, line 6↑).

3. Another potential site of action for ATP8a1 is the TGN and it seemed odd that a TGN marker was not examined for co-localization with ATP8A1 in Fig 1. Perhaps this could be done as well.
> Thank you for the suggestion. We examined the co-localization of GFP-ATP8A1 and a TGN marker TGN46. As shown in revised Fig 1A, GFP-ATP8A1 did not co-localize with TGN46. The corresponding text was revised accordingly (page 6, line 9↑).

4. It is surprising that 100% of the NBD-PS is getting translocated in the reconstitution experiments shown in Fig 4B and that the NBD-PE translocase activity was that high. This is significantly more efficient than published reports raising concerns that the membrane integrity was compromised and the quencher was accessing the luminal leaflet during the assay. The ATP-dead control looks good and so the appropriate control is there. I don't have a specific recommendation other than to alert the authors that this result is a little suspicious.
> Thank you for the comment. In Fig 4B and Fig 7C, the original manuscript reads: “% NBD-Lipid transport” and “% NBD-PS transport” were normalized to “NBD-PS ATP8A1 (WT)” and “WT”, respectively. This way of presentation was misleading as noted by Reviewer 1. Since we had the absolute value of flipped NBD-lipid (%) (about 10 %) as in the previous report (Coleman and Molday, 2011), we showed these values in revised Fig 4B and Fig 7C.

5. In figure 6B it appears that 2XPH labels only the RE, while Lact-C2 labels the plasma membrane and RE. Is this the case? If so, why do the authors think the PS at the plasma membrane is not being recognized by 2XPH?
> Thank you for the comment. As shown in the figure below, 2xPH expressed in the cytosol, like GFP-lact-C2 expressed in the cytosol, labels the PM as well as REs. Therefore, 2xPH certainly recognizes PS at the PM. In contrast to these PS-probes expressed in the cytosol, recombinant 2xPH stain was very faint after fixation and

permeabilization (Fig 6B). We do not know the exact reason for this, but assume that fixation and permeabilization before 2xPH treatment may interfere with the staining of PS at the PM.



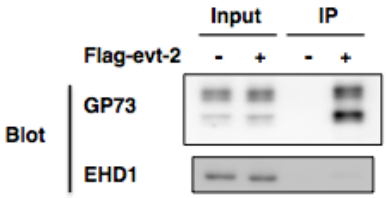
[The expression of GFP-2xPH in the cytosol in COS-1 cells: Arrows indicate the PM localization of GFP-2xPH]

6. In the Chen et al 2010 PLoS Genetics paper, the Wang group localized RME-1 (EHD1 homolog) in the tat-1 mutant. They reported a modest loss of membrane association but most of the RME-1 still localized to abnormal endosomes. In this case, defects in trafficking through the RE were not attributed to a loss of RME-1 function. It would appropriate to comment in the Discussion on this modest discrepancy between the literature and the current work.

> As Reviewer 1 pointed out, the loss of *tat-1* leads to the generation of abnormal endo-lysosomal compartments, suggesting that endocytic cargo sorting and recycling are impaired (Ruaud *et al.*, 2009; Chen *et al.*, 2010). In the *tat-1* mutant, an EHD1 homologue, RME-1, still localized at abnormal endo-lysosomes. We cannot explain at the present time the apparent discrepancy in the localizations of EHD1/RME-1 in the loss of ATP8A1/TAT-1 between mammalian cells and *C. elegans.* The loss of *tat-1* is also known to affect the PS distribution of the PM (Darland-Ransom *et al.*, 2008) as well as endo-lysosomes, and has profound effects on various membranes (Darland-Ransom *et al.*, 2008; Ruaud *et al.*, 2009; Chen *et al.*, 2010), which may result in intracellular organelles with abnormal membrane components such as lipids. RME-1 might be associated with these abnormal membranes in a PS-independent manner. However, we do not think it is appropriate to include this discussion in the manuscript without any circumstantial evidence. We have just commented on the apparent discrepancy in the localization of EHD1/RME1 in the revised Discussion (page 18, line 4↑).

7. What is the functional relationship between evectin-2 and EHD1? Both may be effectors of PS flip by ATP8A1. Do the authors think they are both involved in scission or is evectin-2 doing something else? Does evectin-2 interact with EHD1? Perhaps this could be commented on in the Discussion.

> Thank you for the comments. First, we did not detect the interaction of evectin-2 with EHD1 (see attached figure below) under the conditions where evectin-2 binds to GP73 (Uchida *et al.*, 2011). Second, evectin-2 knockdown impaired the retrograde transport of GP73 to the Golgi, but did not cause tubulation of RE membranes (Uchida *et al.*, 2011). Thus, at the present time, we do not have any evidence for the role of evectin-2 in the EHD1-mediated fission process and therefore would like not to include this issue in the revised manuscript.



[FLAG-tagged evectin-2 was expressed in COS-1 cells for 24 h. Cell lysates were then immunoprecipitated with anti-FLAG antibody. Immunoprecipitates were then blotted with anti-GP73 or anti-EHD1 antibody]

8. From the Discussion - "Thus, although ATP8A1 contributes to the enrichment of PS in the cytosolic leaflet of RE membranes, its knockdown does not result in the complete disappearance of PS at the cytosolic leaflet. Scramblases that mediate bidirectional transbilayer movement of phospholipids or other P4-ATPases may participate in the exposure of PS at the cytosolic leaflet in ATP8A1-depleted cells." PS is synthesized in the cytosolic leaflet of the ER and likely moves by both vesicular and nonvesicular routes to the PM and other organelles. The nonvesicular route would necessarily deliver PS to the cytosolic leaflet and may move the majority of the PS in the cell.
> Thank you for the suggestion. We added the following discussion in the revised manuscript (page 20, line 13):
"PS is synthesized in the cytosolic leaflet of the ER and likely moves via non-vesicular routes (Maeda *et al.*, 2013) and/or vesicular routes to the PM and other organelles. These processes (Holthuis and Menon, 2014) may also contribute to the localization of PS at the cytosolic leaflet of the RE membranes."

9. The title could better reflect the content of the paper. For example "Transport through recycling endosomes requires EHD1 recruitment by a phosphatidylserine translocase".

- We agreed with Reviewer 1's suggestion and changed the title.

Reviewer 2:

Major concerns:
1) In the first figure, the authors discuss "colocalisation" but as they have not quantified any of the data, have no means to determine to what degree this quantification occurs (or does not). In addition, the data provided for the knock-down is really not compelling- the western blot is rather dirty and both siRNA2 and 3 don't appear to show any decrease. Whilst the siRNA1 does show an increase, the loading control of tubulin is also distinctly lower, leaving this reviewer concerned as to whether knock-down is occurring at all.
> Thank you for the important comments. We quantified the colocalization (Pearson's coefficient) between GFP-ATP8A1 and the organelle markers used in the original manuscript. GFP-ATP8A1 showed higher co-localization with RE proteins (Pearson's coefficient with TfnR, 0.78; mCherry-Rab11, 0.89) than the other organelle markers (0.31 – 0.53), suggesting the predominant localization of ATP8A1 at REs. We added the Pearson's coefficient to revised Fig 1A.
We also performed another Western blot for the knock-downs of ATP8A1, and obtained a clearer blot than the original. We confirmed that ATP8A1 siRNA1 was more effective in knockdown of ATP8A1 than siRNA2 or siRNA3. The new blot was included as revised Fig 1B.

2) In the same figure, whereas the Tf recycling seems to be impacted by ATP8A1 knock-down, an important control should be a reinsertion of the wild-type ATP8A1 to show that the effect is not an off-target one.
> Thank you for the critical suggestion. We expressed an siRNA-resistant wild-type ATP8A1 in ATP8A1-depleted cells, and examined the Tfn recycling. We performed a set of experiments using Alexa 594-Tfn for both control and ATP8A1 KD cells (revised Fig 1C) and confirmed the previous conclusion from original Fig 1C and D. Moreover, as shown in revised Fig 1D and Supplementary Fig S2, reinsertion of the wild-type ATP8A1 restored Tfn recycling (green line) almost to the control level (blue line in revised Fig 1C), ruling out the possibility of an off-target effect of siRNA.
We revised the text (page 7, line 1) as follows:
"For the recycling assay, cells were first pulsed with Alexa 594-Tfn and then chased in media without Alexa 594-Tfn. During the chase, cells were imaged every 6 min for 1 h. After the pulse, Alexa 594-Tfn accumulated at REs in both control and siRNA-treated cells. In control cells, the fluorescence intensity of Alexa 594-Tfn in REs decreased by 50% after a 30-min chase and decreased by 70% after a 50-min chase (Fig 1C and Supplementary Fig S2). In contrast, in ATP8A1-depleted cells, the fluorescence intensity of Alexa 594-Tfn in REs decreased by 10% after a 30-min chase and

decreased by 20% after a 50-min chase, suggesting that ATP8A1 is required for efficient recycling of Tfn from REs to the PM. We also performed the rescue experiment by the expression of an siRNA-resistant wild-type ATP8A1. As shown in Fig 1D and Supplementary Fig S2, reinsertion of the wild-type ATP8A1 restored Tfn recycling (not significant versus "Control" in Fig 1C by Student's *t*-test)."

3) The authors relate extensively to the aberrant tubules formed, and even show that ATP8A1 ATPase activity loss is required for their formation. There is little clarification of the significance of these aberrant structures. How are they formed? What are they? Why are they significant? If anything, the knock-down/knock-in experiment with the ATPase-deficient mutant would be better served in the Tf recycling assay noted above.
> Thank you for the critical suggestion. We expressed an siRNA-resistant, activity-deficient mutant (E191Q) in ATP8A1-depleted cells. As shown in revised Fig 1D and Supplementary Fig S2, the expression of the E191Q mutant could not restore Tfn recycling (purple line). Together with the data showing that the E191Q mutant could not reduce the number of cells that had TnfR-positive tubules (Fig 2E), these results suggest that ATPase activity of ATP8A1 is required for Tfn recycling and normal RE morphology.
We revised the text (page 9, line 12) as follows:
"We then expressed an siRNA-resistant WT ATP8A1 or E191Q mutant in ATP8A1-depleted COS-1 cells. The number of cells that had TfnR-positive tubules was reduced by the expression of the siRNA-resistant WT ATP8A1 but not by the expression of the siRNA-resistant E191Q mutant (Fig 2E). The expression of E191Q mutant also could not restore Tfn recycling (Fig 1D). Together, these results suggest that normal RE distribution of TfnR and Tfn recycling depend on the ATPase activity of ATP8A1."

4) I have concerns about the physiological significance of the liposome assays. 40-70% PS is very high, and PE is probably not the best control. Sedimentation or flotation assays with lower concentrations would be a lot more convincing.
> As shown in Fig 5A and C of the original manuscript, a sharp increase of the binding of EHD1 was observed from 40 to 70% PS with half-maximal binding at 53% PS in liposome sedimentation assay. As other reviewer suggested, we examined the effect of the liposome curvature on the binding of EHD1. We found that when the curvature of liposomes increased (*i.e*., the diameter decreased), more EHD1 bound to liposomes at PS concentration lower than 70%. These results are included in revised Fig 5D and E. Thus, the PS concentration required for EHD1 binding to the cytosolic leaflet of RE membrane could be lowered below 70% by the curvature of RE membranes. We commented on these points in the Results section (page 13, line 11 - page 13 line 17).
We estimated the amount of PS in the cytosolic leaflet of REs in the original manuscript as follows: “PS comprises 5 - 10% of the total phospholipids in mammalian cells (Vance and Steenbergen, 2005; Lee et al., 2012). PS is 5-fold more abundant in the purified RE fraction than in the cell lysate (Gagescu et al., 2000), suggesting that PS accounts for 25 - 50% of the total phospholipids in RE membranes. Using two PS-specific probes, we roughly estimated that 70% and 30% of PS are localized in the cytosolic and the luminal leaflets of RE membranes, respectively. Taking this PS asymmetry into consideration, PS may comprise 35% (= 25 x 2 x 0.7) - 70% (= 50 x 2 x 0.7) of total phospholipids in the cytosolic leaflet of RE membranes.” Thus, it may not be surprising that 70% PS exists in the cytosolic leaflet of RE membranes depending on cell type.
We are not certain that PE is the best control for a liposome sedimentation assay. We chose PE because only this phospholipid may exist at such a high concentration in the cytosolic leaflet of the membranes.

5) Another major question left unanswered is how EHD1 binds to PS. Daumke et al. showed that conserved lysines in the helical region of EHD2 are required for membrane sensing and curvature. Are these the lysines that contact PS, allowing charge-based binding? Or is the EH domain (as suggested by Keiken et al.) responsible for this interaction?
> Thank you for the important suggestions. To test if conserved K324 in EHD1 helical

region is required for the PS binding, we performed a liposome sedimentation assay using the recombinant EHD1 (K324D) and found that this mutant did not bind to liposomes that contain 60% PS (Supplementary Fig S13), suggesting that K324, one of the conserved lysines (K324) in the helical domain of EHD1, is required for the PS-dependent EHD1 recruitment to the membranes. We also performed the liposome sedimentation assay using the recombinant EHD1 that lacks EH domain (ΔEH). This mutant could bind to the liposomes, comparable to full-length EHD1 (WT) (Supplementary Fig S13), indicating that EH domain is not essential for PS binding.
We next examined the localization of these mutants in COS-1 cells. GFP-EHD1 (WT) was localized at perinuclear REs and cytoplasmic tubular structures (revised Supplementary Fig S13). These tubular structures may correspond to "tubular recycling compartment" in HeLa cells (Caplan *et al.*, 2002). K324D lost the localization at both membrane structures and was dispersed completely to the cytoplasm, suggesting that K324 in helical region is essential for the membrane localization of EHD1. ΔEH still localized at perinuclear REs, but not at cytoplasmic tubular structures (Supplementary Fig S13). This observation is consistent with the previous report that ΔEH did not localize at tubular recycling compartment in HeLa cells (Caplan *et al.*, 2002). Since ΔEH still localized at perinuclear REs (Supplementary Fig S13), the perinuclear RE localization of EHD1 does not require EH domain.
Since the comment (5) is related to (6), we discussed these results together with the response to (6).

6) Perhaps the most major conceptual concern is how the authors relate their study to that of Giridaran et al. The latter authors presented a model suggesting that EHD1 recruitment to RE occurs via several NPF-containing proteins, Mical and Pacsin, which reportedly bind to phosphatidic acid. It's entirely possible that PS could independently recruit EHD1, regardless of Mical and Pacsin, but it's also entirely possible that PS levels indirectly affect phosphatidic acid or recruitment of Mical and Pacsin. In any case, the authors of this manuscript have not even addressed these issues and this model, either experimentally or conceptually.
> As commented above, K324D mutant did not bind to PS-liposomes *in vitro* and did not localize at perinuclear REs or cytoplasmic tubular structures. Together with the observation that GFP-EHD1 (WT) was completely dispersed from these membrane structures into the cytoplasm in PS-deficient CHO mutants (Fig 4D), these results indicate that binding of EHD1 to the membranes requires PS. On the other hand, ΔEH mutant bound to PS-liposomes *in vitro*, but could not localize at cytoplasmic tubular structures, suggesting that PS is not the sole determinant for EHD1 localization at cytoplasmic tubular structures. As Reviewer 3 pointed out, recruitment of EHD1 to tubular structures in HeLa cells requires the interactions between EH domain and NPF-containing proteins, such as MICAL-L1 (Sharma *et al.*, 2009) and Pacsin (Giridharan *et al.*, 2013). It is also proposed that MICAL-L1 and Pacsin bind to phosphatidic acid in the tubular structures (Giridharan *et al.*, 2013). We agree with the idea that EHD1 binds to tubular structures through MICAL-L1/Pacsin/phosphatidic acid. It is tempting to speculate that there are two distinct recycling compartments, perinuclear REs to which EHD1 is recruited primarily by PS and tubular recycling compartment to which EHD1 is recruited cooperatively by phosphatidic acid and PS.
We revised the discussion based on these observations (revised Supplementary Fig S13) (page 20, line 7↑ - page 21 line 7↑) as follows:
“EHD proteins contain an amino-terminal ATP-binding G-domain, followed by a helical domain and a carboxy-terminal EH-domain (Grant and Caplan, 2008). The crystal structure of full-length EHD2 was solved (Daumke *et al.*, 2007). Dimerization of G-domain creates a putative membrane-binding surface with a polybasic stretch within the helical domain (residues 285 - 400). EHD2 mutants (F322A, K324D, K327D, K328D, or K329D) display a completely cytoplasmic distribution, showing the critical role of these amino acids in the helical domain in membrane localization of EHD2 (Daumke *et al.*, 2007). EHD1 (K324D) lost its membrane localization in COS-1 cells similarly to EHD2 (K324D), and did not bind to PS-liposomes *in vitro* (Supplementary Fig S13), which supports the idea that PS is essential for binding of EHD1 to the membranes. We noticed that when expressed in COS-1 cells, GFP-EHD1 localized at perinuclear REs and cytoplasmic tubular structures (Supplementary Fig S13). The latter

was not obvious when endogenous EHD1 was immuno-stained (Fig 3). The tubular structures may correspond to "tubular recycling compartments" in HeLa cells (Caplan *et al.*, 2002). EHD1 that lacks EH-domain (ΔEH) still localized at perinuclear REs, but not at cytoplasmic tubular structures (Supplementary Fig S13). This observation is consistent with the previous report that ΔEH did not localize at tubular recycling compartment in HeLa cells (Caplan *et al.*, 2002). However, ΔEH mutant bound to PS-liposomes *in vitro*. These results suggest that PS is not the sole determinant for EHD1 localization at cytoplasmic tubular structures. Recruitment of EHD1 to tubular structures in HeLa cells requires the interactions between EH domain and NPF-containing proteins, such as MICAL-L1 (Sharma *et al.*, 2009) and Pacsin (Giridharan *et al.*, 2013). It is also proposed that MICAL-L1 and Pacsin bind to phosphatidic acid in the tubular structures (Giridharan *et al.*, 2013). It is tempting to speculate that there are two distinct recycling compartments, perinuclear REs to which EHD1 is recruited primarily by PS and tubular recycling compartment to which EHD1 is recruited cooperatively by phosphatidic acid and PS."

Reviewer 3:

1. It is important to show the raw data for the flippase assay using purified, reconstituted proteins. This is important as the bar chart shown in Figure 4B obscures the fact that the activity signals in these flippase assays are typically very small and can be unconvincing.
> As Reviewer 3 pointed out, the way of presentation was misleading in the original manuscript. Since we had the absolute value of flipped NBD-lipid (%) (about 10 %) as in the previous report (Coleman and Molday, 2011), we showed these values in revised Fig 4B and Fig 7C.

2. Recent reviews by Bigay and Antonny (Dev Cell 2012) and Holthuis and Menon (Nature 2014) could be cited as they discuss the role of PS on the cytoplasmic face of membranes of the late secretory pathway. The latter review also discusses the regulation of P-type ATPases, something of relevance to the present paper.
> Thank you for the suggestion. We cited these two references in the revised texts as follows: "Recent studies have revealed the significance of PS in the cytosolic leaflet of endosomes [reviewed in (Bigay and Antonny, 2012; Holthuis and Menon, 2014)] (page 21, line 6↑).
As Reviewer 3 pointed out, the regulation of P-type ATPases is an important issue. So far, we do not have any data on the regulation of ATP8A1. In yeast, PI(4)P activates Drs2p in the TGN, and this activation is essential for the secretory traffic from the TGN. We have just cited this work (Natarajan *et al.*, 2009) and the review (Holthuis and Menon, 2014) in the revised manuscript (page 22, line 9↑).

3. The authors should comment briefly in the discussion section on the enigmatic presence of ATP8a2 in disc membranes of photoreceptor cells. What would be the role of this protein in discs given its proposed role in the secretory pathway?
> Thank you for the suggestion. We added the following discussion on ATP8A2 in the revised texts (page 23, line 5):
"ATP8A2 in photoreceptor cells is localized both in the perinuclear compartment of the inner segment and in the disc membranes of the outer segment (Coleman *et al.*, 2009, 2014). ATP8A2 in the perinuclear compartment may produce the lipid asymmetry in the bilayer to facilitate vesicle trafficking to the disc membrane, which could explain a drastic reduction in the length of outer segments in ATP8A2-deficient mice (Coleman *et al.*, 2014). Since disc membranes have a symmetrical distribution of PE and PS (Wu and Hubbell, 1993; Hessel *et al.*, 2000), presumably due to significant scramblase activity of rhodopsin (Menon *et al.*, 2011), ATP8A2 does not appear to play a role in generating global lipid asymmetry in disc membranes. Some portion of ATP8A2 may be transported to the disc membranes as a result of vesicle trafficking from the perinuclear compartment."

2nd Editorial Decision 12 December 2014

Thank you for submitting the revised version of your manuscript entitled 'Transport through recycling endosomes requires EHD1 recruitment by a phosphatidylserine translocase'. I have now received reports from two of the original referees, who now support publication pending minor revision.

Referee #2 still raises a few concerns, and I would like to ask you to address these concerns in a point-by-point response and in your discussion. I realize that your discussion includes the rationale of using liposomes with a very high PS content, but maybe you could add this explanation also in the relevant part of the results section. Please note that we do not require further experiments for publication at this stage.

A few editorial points need to be taken care of at this stage as well:

- Please upload a modified manuscript text file.
- Please check whether all figure files are of adequate resolution and quality for production, and upload improved versions if necessary.
- We also encourage our authors to provide original source data, particularly uncropped/-processed electrophoretic blots for the main figures of the manuscript. If you would like to add source data, we would welcome one PDF-file per figure for this information. These will be linked online as supplementary "Source Data" files.
- Please suggest (in a cover letter) 2-5 one-sentence 'bullet points', containing brief factual statements that summarize key aspects of the paper - they will form the basis for an editor-drafted 'synopsis' accompanying the online version of the article. Please see the latest research articles on our website (emboj.embopress.org) for examples - I am happy to offer further guidance on this if necessary.
- Finally, it would also be great if you could provide a very basic model figure to be used for the synopsis. The size would have to be within the format restrictions of 550 pixels (width) x 150-400 pixels (height).

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

REFEREE REPORTS

Referee #1:

The authors have done an exemplary job of responding to my prior critique and I believe this manuscript represents a substantial advance in the membrane biology and protein trafficking fields. The quality of the data is quite good and the conclusions are very well supported, typically by several different approaches. The studies clearly establish that PS translocation by a P4-ATPase in the recycling endosome is crucial for protein trafficking through this organelle. Convincing data also link this activity to EHD1 function at the RE providing important new mechanistic insight. I believe this to be an outstanding contribution and extend my congratulations to the authors.

Referee #2:

The authors have provided a much improved manuscript following this round of revision. There remain several minor issues that would be best clarified.

1) Although ATP8A1 KD did not affect EGF trafficking (control in Fig. S3A and B), the lysosomes themselves appear to display somewhat different morphology. They appear larger and more ring-like. Is this a true phenotype, or merely that the micrographs were obtained from a different plane of focus?

2) The tubules that appear to emanate from the RE/ERC region upon ATP8A1 are intriguing,

because they are reminiscent of the enhanced tubulation noted by Cai et al. (MBoC 2012 and JBC 2013) upon EHD1 depletion. Do the authors think these structures are EHD3/MICAL-L1-positive?

3) Despite the rationale and explanations that PS is likely to be highly enriched in RE/ERC, as noted by this and other reviewers, liposome PS concentration is nonetheless very high in the pulldown assays. One concern is the negative charge of the PS (compared to PE, for example). I think that adding similarly high concentrations of PIP2 and demonstrating selectivity of binding for EHD1 might help convince this reviewer of the specificity of binding.

4) On p. 8, please check the text: should "at the mixture" be "as a mixture" and "localized to" rather than "localized at."

2nd Revision - authors' response 14 December 2014

Reviewer 2:

1. Although ATP8A1 KD did not affect EGF trafficking (control in Fig. S3A and B), the lysosomes themselves appear to display somewhat different morphology. They appear larger and more ring-like. Is this a true phenotype, or merely that the micrographs were obtained from a different plane of focus?
> As Reviewer 2 pointed out, ATP8A1 knockdown affected the lysosomal morphology. LAMP1-positive lysosomes appeared consistently swollen in ATP8A1-depleted cells. Since ATP8A1 did not localize at lysosomes, we assume that the phenotype arises as the secondary effect of the impaired membrane traffic from REs. We briefly commented on the phenotype in the "Result" of the revised manuscript (page 7, line 4↑) as follows:

"After a 5-min pulse / 60-min chase of EGF, a portion of EGF co-localized with LAMP1 in control and ATP8A1-depleted cells. Although the LAMP1-positive lysosomes appeared swollen in ATP8A1-depleted cells, no significant difference in the Pearson's coefficient between LAMP1 and EGF was observed (Supplementary Fig S3A and B)."

2. The tubules that appear to emanate from the RE/ERC region upon ATP8A1 are intriguing, because they are reminiscent of the enhanced tubulation noted by Cai et al. (MBoC 2012 and JBC 2013) upon EHD1 depletion. Do the authors think these structures are EHD3/MICAL-L1-positive?
> Thank you for the suggestion. Aberrant tubules in EHD1-depleted HeLa cells found by Cai *et al*. was characterized as CD59-positive structure, while the tubules in ATP8A1 or EHD1-depleted COS-1 cells were characterized as TfnR-positive structure. Thus, it is not clear if these tubular structures have the same endosomal origin, and we do not have data, at the present time, that EHD3 and/or MICAL-L1 localized to the aberrant tubules in ATP8A1-depleted cells. We would like to examine this interesting issue in the future study. We think that it is appropriate to cite the two papers by Cai *et al*. in the "Introduction" of the revised manuscript (page 5, line 3) as follows:

"In EHD-1 depleted cells, CD59-positive endosomes became tubulated (Cai *et al.*, 2012, 2013)."

3. Despite the rationale and explanations that PS is likely to be highly enriched in RE/ERC, as noted by this and other reviewers, liposome PS concentration is nonetheless very high in the pulldown assays. One concern is the negative charge of the PS (compared to PE, for example). I think that adding similarly high concentrations of PIP2 and demonstrating selectivity of binding for EHD1 might help convince this reviewer of the specificity of binding.
> We did the experiment on the binding of EHD1 to PC-based liposomes that contain 60% $PI(4,5)P_2$. As shown in the revised Supplementary Fig S9A, the significant amount of EHD1 bound to the liposomes. Therefore, the binding of EHD1 to PS-liposomes is not necessarily through the PS structure-specific interaction, but rather through the electrostatic interaction. We added the result of liposome co-sedimentation assay with 60% $PI(4,5)P_2$ in the "Result" in the revised manuscript (page 13, line 10):

"EHD1 did not bind to liposomes that contain 60% PE (Fig 5A and B), but bound to liposomes that contain 60% $PI(4,5)P_2$ (Supplementary Fig S9A), suggesting that the binding of EHD1 to PS-liposomes is not necessarily through the structure-specific interaction, but rather through the electrostatic interaction."

4. On p. 8, please check the text: should "at the mixture" be "as a mixture" and "localized to" rather than "localized at."
> Thank you for the correction. The corresponding text was revised accordingly (page 8, line 6↑ - line 3↑).

3rd Editorial Decision 16 December 2014

Thank you very much for sending the revised version of your manuscript and the point-by-point response, as well as the synopsis and bullet points. I am happy to inform you that your manuscript is accepted in principle for publication here.

