Retromer retrieves the Wilson disease protein ATP7B from endolysosomes in a copper-dependent manner

Santanu Das, Saptarshi Maji, Ruturaj, Indira Bhattacharya, Tanusree Saha, Nabanita Naskar and Arnab Gupta
DOI: 10.1242/jcs.246819

Editor: Mahak Sharma

Review timeline
Original submission: 29 March 2020
Editorial decision: 1 May 2020
First revision received: 28 October 2020
Editorial decision: 16 November 2020
Second revision received: 18 November 2020
Accepted: 19 November 2020

Original submission

First decision letter

MS ID#: JOCES/2020/246819

MS TITLE: Retromer retrieves the Wilson Disease protein, ATP7B from lysosomes in a copper-dependent mode

AUTHORS: Arnab Gupta, Santanu Das, Saptarshi Maji, Rutu Raj, Indira Bhattacharya, and Tanusree Saha
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. In particular, several methodological shortcomings, incomplete analyses and controls have been pointed out by the reviewers. A more detailed analysis of the late endosomal/lysosomal compartment using specific markers and probes is required as well as rigorous analysis of the imaging data with description in the methods should be added. Based on these considerations, and in spite of the general interest of the topic, I cannot accept your manuscript at this stage.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

Please provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

"Retromer retrieves the Wilson Disease protein, ATP7B from lysosomes in a copper-dependent mode"

Das et al.,

In this study, the recycling of the Copper Transporter protein, ATP7B is studied and a role for retromer is reported in the recycling of ATP7B from 'lysosomes' to the Golgi. The ATP7B protein is mutated in Wilson's disease that is caused by a failure of copper homeostasis.
It is now well established that the retromer complex functions in endosomes to Golgi retrieval of a variety of membrane proteins being first described in this role from studies conducted in yeast published more than 20 years ago. It has also been demonstrated that retromer, along with the WASH complex and accessory/adaptor proteins such as Snx27 or the COMMD-containing CCC complex is necessary for the endosomes to cell surface recycling of proteins such as integrins, the glut1 protein and also the related ATP7A copper transporter.
The study by Das and colleagues therefore adds to a growing body of literature detailing a role for retromer in endosomal protein sorting to either the Golgi or the cell surface. Certainly there is nothing profoundly game-changing in this study but understanding the trafficking of important proteins such as the ATP7B transporter is a worthy pursuit. I think that, with suitable revision it may be appropriate for publication in J Cell Science but some significant changes to the text are required along with additional experimental data and clarification of the methods applied.

Comments for the author

The study and the manuscript come across as somewhat naive and simplistic at times and there are various revisions I believe are necessary.

Major points:

1. The study seems to take a very narrow view on what a lysosome is and has rather arbitrarily chosen Lamp-1 positive status to define a lysosome. It is now well accepted that lysosomes are more dynamic entities than previously envisioned. There are 'lysosomes' that can be positive for hydrolases such as cathepsin D but not necessarily active. Similarly, there are 'lysosomes' that have markers of endosomes present where active hydrolyses are also localised. I would suggest that the author should read and assimilate the data presented in Bright et al., 2016 (PMID: 27498570) which shows how the lines between endosomes and lysosomes have become increasingly blurred.
2. Are the Lamp-1 positive 'lysosomes' shown in Das et al., also positive for hydrolases (e.g. cathepsin D)
and active hydrolases? This requires the use of the Magic Red dye that becomes fluorescent after cleavage by cathepsin B. Are other 'markers' of lysosomes present such as TFEB? The data presented in the study by Das et al., regarding the identity of the 'lysosomes' they show is currently rather superficial and needs extending.
3. The text should be changed so that the basic message of the manuscript is that retromer mediates the retrieval of the ATP7B protein from endolysosomes as this term is a more encompassing way of describing the organelles where retromer is functioning in this study.
4. I have concerns regarding how the imaging was performed. When the images were collected was this performed 'blind'? Did the person imaging know what conditions the cells were treated with? Much of the data presented is in the form of immunofluorescence images and their subsequent quantification but if there is any bias (conscious or unconscious) in the imaging process then the

data may be unreliable along with any conclusions drawn from the data. Selected experiments should be repeated but imaged under
'blind' conditions or the methods section should be revised to show what steps were taken to prevent any bias during imaging.
5. Can the complexes created by crosslinking ATP7B to retromer be better defined? Perhaps the complexes could be isolated by immunoprecipitation and then analysed by mass spectrometry. This would potentially reveal if other proteins (e.g. a sorting nexin) are also involved with retromer in mediating ATP7B recycling.
6. How does loss of SNX3 or RAB7A affect ATP7B recycling? Both these proteins are required for retromer membrane recruitment and/or association and loss of SNX3 or RAB7A would be expected to phenocopy loss of VPS35. How does loss of the COMMD-containing complex affect ATP7B recycling? It has been shown that ATP7A traffic to the cell surface requires the COMMD complex (see PMID: 25355947) so it would be interesting to know if ATP7B is similarly impacted by loss of COMMD function.
7. The authors frequently do not cite the literature correctly and need to make some significant changes to ensure the literature is correctly cited. For example, the authors cite Arighi et al., 2004 with respect to CIMPR retrieval but not Seaman 2004 that is in the same edition of JCB and also reports the requirement for retromer in CIMPR recycling. The authors should read and cite the studies from the Burd laboratory on the trafficking of the yeast iron transporter as they are pertinent to the study by Das et al on ATP7B trafficking (see PMID: 17420293 and others). They should also cite the Burd-lab study on the recruitment of retromer by RAB7A and cargo proteins (see PMID: 24344282). There are a great many other examples where the literature is incorrectly cited and I would urge the authors to actually read the retromer literature rather than relying only on the title of manuscripts published.
8 What is BCS? I could find nothing substantive in the methods section to detail the treatments of the cells with copper or chelating agents etc.
9. The introduction and the discussion are both too long and wordy and lack focus and should be revised accordingly so that they respectively frame the question and then assimilate the data into what is currently known about retromer and ATP7B recycling.

Reviewer 2

Advance summary and potential significance to field

This manuscript analyzes of the role of retromer in the trafficking of copper-transporting ATPase ATP7B, whose mutations cause inherited disorder of copper metabolism known as Wilson disease. Relocation of ATP7B from the Golgi to late endosomes and lysosomes is required for sequestration of excess and, hence, potentially toxic copper, while reduction in copper levels stimulates retrieval of ATP7B to the Golgi, where it metalates several copper-dependent proteins. Although anterograde trafficking of ATP7B was thoroughly dissected the mechanisms driving retrieval of ATP7B to the Golgi remain poorly understood. Here the Authors employed a combination of biochemical and cell biology approaches to demonstrate that VPS35 drives copper-dependent trafficking of ATP7B from late endo-lysosomal vesicles to the Golgi. Altogether, the main findings of the paper provide new important insights in understanding of ATP7B trafficking mechanisms and their possible implication in Wilson disease and other pathologies. Therefore, the manuscript might be of great interest to the readership of the Journal of Cell Science.

Comments for the author

In my view, in its current form the manuscript needs revision to address the below comments.

1) It remains unclear whether and how VPS35-mediate trafficking of ATP7B contributes to maintenance of copper homeostasis. Does VPS35 silencing affects (i) intracellular copper levels, (ii) tolerance of the cells to copper or (ii) activity of ATP7B? This issue is of fundamental importance for understanding of the mechanisms behind the regulation of copper balance in health and disease and requires additional experiments to be clarified. Tolerance to copper might be easily evaluated with live-dead staining, labelling of apoptotic cell/nuclei and MTT assay. ATP7B activity might be evaluated with tyrosinase/L-DOPA method, which was used by corresponding author in several publications.

2) Discussion. Recent JCS publication (Stewart et al., 2019) shows that the retrograde ATP7B trafficking might be also regulated by COMMD1, whose silencing results in increased colocalization of ATP7B with VPS35. COMMD1, as a part of CCC complex, has been shown to cooperate with VPS35 in retrograde trafficking of ATP7A. I think that this should be discussed especially in the context of "large" protein complex that might bridge VPS35 to ATP7B.

3) Discussion. The authors say that they never observed ATP7B at the plasma membrane of HepG2 cells (even treated with copper). It would be probably worth to specify that HepG2 cells were not polarized in this study, because in polarized HepG2 cells ATP7B is detectable at the apical surface.

4) Page 8. The Authors say that they confirmed the role of retromer by rescuing the non-recycling phenotype of ATP7B in VPS35 kd cells by overexpressing wt-VPS35. I suggest testing VPS35-R107A in rescue experiments. According my expectations, it should not rescue non-recycling phenotype of ATP7B or at least should not be as efficient as wt-VPS35.

5) Western blots in figures 2D, 4A, 5A, and S2B lack input marker (GAPDH, actin or tubulin), which has to be provided.

Reviewer 3

Comments for the author

Copper is a red-ox metal and acts as an essential micronutrient for all cells. Intracellularly, copper homeostasis is maintained through two different transporters, ATP7A and ATP7B, wherein ATP7A expresses ubiquitously and ATP7B is limited to liver, brain and kidney. Mutations or defective function of these proteins results in Menkes disease and Wilson disease, respectively. Both proteins localize to Golgi (majorly to TGN) and supply the copper to the biosynthetic/secretory cuproproteins. ATP7A maintains copper levels by cycling between TGN and cell surface depending on its intracellular concentration. In contrast, ATP7B traffic to lysosomes from TGN at elevated copper levels; and the lysosomes dispose the copper to extracellularly through lysosomal exocytosis. However, it is not clear whether ATP7B can recycle back to TGN from lysosomes during the reversal of high to basal concentration of copper. Moreover, the mechanism behind this process is not well studied. In this study, authors established that recycling of ATP7B from endolysosomes or LAMP-1-positive compartments to TGN requires retromer complex consisting VPS35, 26 and 29. Using high end imaging, UV-crosslinking and biochemical interaction studies, authors showed that ATP7B localize juxtaposed and interacts with VPS35 at high concentration of copper availability. Further, retromer knockdown experiments demonstrated that the complex is required for retrograde transport of ATP7B from LAMP-1-positive compartments to TGN. This study is quite unique and highlighted that the transporter ATP7B can recycle back to TGN through retromer for next round of copper transport. However, authors should consider reviewing the below suggestions, which may improve their manuscript.

Major comments:

1. Authors indicated that ATP7B recycles from lysosomes in response to copper. However, it is not clear from their studies that the organelles are late endosomes or lysosomes or endolysosomes. In majority of the time authors used either Rab7 or LAMP1 as a marker; and indicated that those are lysosome (including the manuscript title). Authors should consider to use dextran uptake to distinct the terminal lysosomes vs endo-lysosome intermediates. Moreover, even in their model figure they did not indicated the nature of the organelles and the structures look like MVBs/recycling endosomes.

Also note that authors concluded that retromer sorts ATP7B from lysosomes and late endosomes (line 104); however, the title and abstract say only lysosomes. Earlier point is consistent with their SIM data that ATP7B localizes to late endo-lysosomal compartments (see line 141, Fig. 3).

2. In Figure 1A (panel 2), the Golgi (Golgin97 staining) appears to be dispersed upon treatment of cells with 50 ¤M for 2 h. Authors can explain the reason for this TGN/Golgi dispersal? Instead of overlaps plots (or in addition to these plots), authors should consider including the inset in the figure to emphasize the colocalization.

3. Authors showed colocalization of ATP7B with LAMP1 (r=>0.5, equivalent to TGN, in Fig. 1B) and Rab7 (r=<0.1) post copper treatment (lines 120-121). Author should note that significant amount of LAMP1 also present in endocytic compartments including Golgi/associated vesicles.

Thus, labeling the lysosomes with other markers such as LAMP-2 or Arl8b or labeled dextran (post uptake) will provide better conclusion to their studies.
In Fig. 2: 1st row-the localization of ATP7B (see 1st panel) appears to be slightly different than Fig. 1 (1st panel) post BCS treatment. 3rd row- large number of cells still showing colocalization with LAMP1 post rescue of copper treatment.
In Fig. S1A: Upon BCS treatment, the localization of ATP7B appears to be dispersed (supposed to be intact and should be in TGN? Moreover, Rab7 staining is very variable between the panel of Fig. S1A and in 3rd row the staining intensity is increased. Consistently, the ATP7B with Rab7 colocalization values are very low compared LAMP1. Similarly, Rab11 staining in the cells is variable and appears to be background staining. Authors should revisit this data either by using Rab7 constructs for anti-Rab7/labelled Transferrin uptake or anti-transferrin receptor antibody for anti-Rab11.

4. Authors showed degradation of ATP7B post 250 ¤M copper treatment (line 123, Fig. 2D). This data requires some controls: (a) should use proteasomal (MG132) and lysosomal (bafilomycin or other) inhibitors during the experiment- this helps to know the major degradative route of ATP7B; (b) should considered use some internal protein control (GAPDH/tubulin/actin) instead of protein estimation for normalization; (c) should considered to test whether LAMP-1 will also change during the treatment conditions.

5. Authors performed SIM studies to distinguish ATP7B localization with Rab7/LAMP1 compartments. Their studies indicated that 44.9% of ATP7B with Rab7-LAMP1-hybrid compartments; around 4% of ATP7B with either Rab7 or LAMP1. However, their error bars are bigger than this difference? Based on this, authors concluded that ATP7B largely recycle from non-degradative compartments (see line 138). **Without** any experimentation, authors cannot conclude those ATP7B-positive compartments are of **non-degradative** nature. To prove this, authors have to use reporter assays using DQ-BSA or magic red. Another possibility may be of “enlarged endosomes/endo-lysosomes” (small cohort of Rab7 and LAMP1 also present in them), consistent with their measured sizes (20-200 µM, diameter).

6. Authors used VPS35 (WT/R107A) overexpression or its knockdown to study the role of retromer in regulating ATP7B recycling. As a positive control, have the authors validated the trafficking of M6PR in any of these conditions?

7. Model (Fig. 8): Authors are not explained/discussed their model in the text? In the model, authors are showing that ATP7B recycles from “ENDOSOME” (not lysosomes)?

8. For discussion: Any model/mechanisms that triggers the ATP7B recycling back to TGN from LEs/endolysosomes or how the high copper treatments hold the ATP7B protein on LEs/endolysosomes rather than its internalization to ILVs following degradation?

9. In discussion: The luminal loops of ATP7B between the 8 TM domains are small and probably escapes lysosomal hydrolases (line 278). This hypothesis may not correct. Since, ATP7B may follow the same trafficking route similar to LAMP1 protein (or) ATP7B may not ubiquitinated/not sorted into ILVs by ESCRT machinery. However, authors observed that ATP7B degradation at 250 µM (2 h) treatment condition (line 293). This may be due to induced luminal changes in endosomes with high copper that probably lead to ATP7B sorting to ILVs following lysosomal degradation.

10. Authors showed high resolution deconvolved image of cells visualizing the colocalization between ATP7B, LAMP1 and VPS35 (see Fig. 7). In this image, authors should consider counting

the number of these clusters in respect to LAMP1-positive organelles. This probably provide an additional information that the retromer dependent sorting of ATP7B possibly occurs from a cohort of Rab7-LAMP1-hybrid organelles.

Minor comments:

1. Better running title: Retromer regulates retrograde trafficking of ATP7B

2. Typos: ***t****rans* Golgi **n**etwork (line 37); P-type ATPase**s** (line 38); remove Trans Golgi Network (line 46); e**x**ports copper **into** lysosomes (line 49); remove 1455 as superscript on AA (line 74); replace trans-Golgi network to TGN (line 108); Fig. 2, C and D – change it to Fig. 2, D and E (line 123); G**FP** (line 195); remove **used as** (duplicated in line 250); **anti-**VPS26 (line 261); and through lysosomes **"in hepatocytes"** (line 270);

3. Line 47: ceruloplasmin- has not been shown to localize to the lumen of TGN?

4. Line 70: di/tri leucine motif – it should be dileucine/acid dileucine motif

5. Line 74: ATP7A to be important for its "retrieval" from cell membrane – 'endocytosis' is the better word

6. Line 89: Authors should note that SNX17 has been shown to form a complex with CCC and WASH complexes, known as 'retriever', regulates retromer-independent endosomal cargo recycling to plasma membrane (PMID: 28892079)

7. Fig. 3: Authors should highlight the source of inset in the main panel and label the inset with respective fluorophore markers.

8. Line 153: CI-M6PR recycles to TGN from "lysosomes" in a retromer regulated fashion. Note that both references clearly suggest that CI-M6PR recycles from **"endosomes"**

9. Line 166: Based on immunoblotting analysis, authors indicated that copper **incubation** does not alter abundance of VPS35 in HepG2 cells. Have the authors noticed any change in membrane recruitment of VPS35 during their conditions?

10. Fig. S2: (B) include some internal control to the blot (also to Fig. 4A, Fig. 5A); (C) Rab7-compartments are smaller than ATP7B punctate structures?

11. Fig. 7: A-D Label the image with respective proteins. Similarly in Fig. S3

12. Have the authors used Ceruloplasmin as positive control in their GFP-ATP7B IP (Fig. S4)?

13. In Fig S1, authors indicated that Rab11 compartments as **mid**-endosomes. Better to label these organelles as recycling endosomes (REs).

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:

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Das et al.,

In this study, the recycling of the Copper Transporter protein, ATP7B is studied and a role for retromer is reported in the recycling of ATP7B from 'lysosomes' to the Golgi. The ATP7B protein is mutated in Wilson's disease that is caused by a failure of copper homeostasis.

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Reviewer 1 Comments for the Author:

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Major points:

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Response: Authors thank the reviewer for the constructive criticism and the suggestions. Using the protocols mentioned by Bright et al (Curr Biol 2016), to distinguish between active acid hydrolase positive endolysosomes and terminal lysosomes we have identified that ATP7B primarily colocalizes with the active lysosomes (Fig. 2C). There is a smaller fraction that localizes on the terminal ones as well (Fig 2C & D). Further, we agree that Lamp1 cannot be an arbitrary marker to label lysosomes/endolysosomes. We have additionally incorporated colocalization assay with Lamp2 and Rab7 as further confirmation of endo-lysosomal localization of ATP7B. The changes and additions are in FigS1 and FigS2.

2. Are the Lamp-1 positive 'lysosomes' shown in Das et al., also positive for hydrolases (e.g. cathepsin D) and active hydrolases? This requires the use of the Magic Red dye that becomes fluorescent after cleavage by cathepsin B. Are other 'markers' of lysosomes present such as TFEB? The data presented in the study by Das et al., regarding the identity of the 'lysosomes' they show is currently rather superficial and needs extending.

Response: We absolutely agree with the reviewer that the identity of the lysosomes harboring ATP7B should be established. As per reviewer's advice, we have labeled the lysosomes with Dextran using the well-established Dex-uptake assay. In parallel we have labeled the active hydrolase positive compartments using the 'Magic red' assay. We have determined the distribution of ATP7B among these two-type of compartments (Dextran-only/terminal lysosome and Dextran-Magic Red/ active hydrolyase positive endolysosome) in high copper condition. We have used two concentration of copper, i.e., 50 μM and 250 μM. interestingly, we found ATP7B to localize in both these compartment types in both copper concentrations with a higher abundance on active lysosomes (Fig2 and FigS4). Upon triggering the retrograde pathway in presence of cyclohexamide, we found ATP7B to return back to TGN under both 50 and 250 μM copper condition. We now have

a clear idea about localization of ATP7B in 'active endolysosomes' vs 'terminal lysosomes in high copper (text added in line: 137-161). Video1 provides a real-time view of fusion/fission of GFP-ATP7B with/from the active endolysosomes.
We agree with the reviewer that we should include an additional marker for lysosome. We have added Lamp2 as a lysosomal marker in addition to Lamp1 (FigS1). ATP7B colocalizes with Lamp2 positive vesicles in high copper that is higher compared to copper chelated condition.

3. The text should be changed so that the basic message of the manuscript is that retromer mediates the retrieval of the ATP7B protein from endolysosomes as this term is a more encompassing way of describing the organelles where retromer is functioning in this study.

Response: We thank the reviewer for the suggestion. We have modified the text and the title accordingly

4. I have concerns regarding how the imaging was performed. When the images were collected was this performed 'blind'? Did the person imaging know what conditions the cells were treated with? Much of the data presented is in the form of immunofluorescence images and their subsequent quantification but if there is any bias (conscious or unconscious) in the imaging process then the data may be unreliable along with any conclusions drawn from the data. Selected experiments should be repeated but imaged under 'blind' conditions or the methods section should be revised to show what steps were taken to prevent any bias during imaging.

Response: We have taken utmost care and precaution while obtaining the data. All the imaging experiments were repeated at least thrice. There was no bias in the imaging process. For all the triplicates of an experiment, the gain and the laser power was set at a constant level. This was a blind study as the imaging and data analysis was done by a different person than the one who prepared the microscopy slides. In the present version we have included the number of cells (n) that were considered while obtaining statistically significant/insignificant data (Figs. 1, 2, 3, S1 and S2).

5. Can the complexes created by crosslinking ATP7B to retromer be better defined? Perhaps the complexes could be isolated by immunoprecipitation and then analysed by mass spectrometry. This would potentially reveal if other proteins (e.g. a sorting nexin) are also involved with retromer in mediating ATP7B recycling.

Response: The crosslinking study reveal that the retromer core proteins interacts with ATP7B and it forms a higher order complex that was stabilized with in-cell crosslinking. In addition we have substantiated this inference using Proximity Ligation assay (PLA). Unfortunately, due to lack of access to some key facilities at this time because of pandemic, we have not been able to use mass spectrometry to further analyze this large complex. However, we have identified that Rab7 regulates the retrograde trafficking of ATP7B in a similar fashion as the retromer core proteins (Fig4, Di). So we can mention that Rab7 is possibly a part of the larger complex. Using FRET-based assay, (Priya et al Traffic 2015, 16: 68–84) and immunoprecipitation (Rojas et al, J Cell Biol 2008, 183(3): 513–526.) have demonstrated that Rab7 interacts with VPS35 and 26 and recruits the retromer complex on the endosomal membrane.

Additionally, we identified that COMMD1 regulates ATP7B retrograde trafficking in a fashion similar to retromer. COMMD1 is linked to the WASH complex that interacts with the retromer complex. So it is highly likely that these proteins assemble on endolysosomal surface as a large super-complex and regulate trafficking of ATP7B. We are as well interested to identify the sorting nexins (SNX) and their mechanism to regulate trafficking of ATP7B. Presently we are putting a serious effort on that study.

6. How does loss of SNX3 or RAB7A affect ATP7B recycling? Both these proteins are required for retromer membrane recruitment and/or association and loss of SNX3 or RAB7A would be expected to phenocopy loss of VPS35. How does loss of the COMMD-containing complex affect ATP7B recycling? It has been shown that ATP7A traffic to the cell surface requires the COMMD complex (see PMID: 25355947) so it would be interesting to know if ATP7B is similarly impacted by loss of COMMD function.

Response: We thank the reviewer for the comment and the suggestion. Using colocalization assay we have determined that Rab7 colocalizes with ATP7B (Fig.S2A and Fig.S3) and the colocalization coefficient increases with increasing copper. Further, as per reviewers suggestion we have compared the effect of mCherry-wtRab7 and dominant mutant mCherry-T22N-Rab7 upon retrograde trafficking phenotype of ATP7B upon copper chelation (Fig. 4Ei). Similar to the VPS35 KD condition, ATP7B stays vesicularized upon copper chelation in cells overexpressing the mutant Rab7 (T22N).

We studied the effect of COMMD1 on ATP7B's anterograde and retrograde trafficking. We utilized GFP-wtCOMMD1 and the two mutants, T174M and K167/173E (Stewart et al, J Cell Sci. 2019 Oct 9;132(19). We noticed that similar to as in VPS35 KD condition, ATP7B stays vesicularized upon copper chelation in cells overexpressing the COMMD1 mutants. In cells overexpressing the wtCOMMD1, ATP7B juxtaposed to a tight perinuclear location indicating its return to the Golgi (Fig. 4Eii).

7. The authors frequently do not cite the literature correctly and need to make some significant changes to ensure the literature is correctly cited. For example, the authors cite Arighi et al., 2004 with respect to CIMPR retrieval but not Seaman 2004 that is in the same edition of JCB and also reports the requirement for retromer in CIMPR recycling. The authors should read and cite the studies from the Burd laboratory on the trafficking of the yeast iron transporter as they are pertinent to the study by Das et al on ATP7B trafficking (see PMID: 17420293 and others). They should also cite the Burd-lab study on the recruitment of retromer by RAB7A and cargo proteins (see PMID: 24344282).
There are a great many other examples where the literature is incorrectly cited and I would urge the authors to actually read the retromer literature rather than relying only on the title of manuscripts published.

Response: We thank the reviewer for pointing out the errors. We have modified the sections that lacked correct references. We have now carefully cited the studies that are relevant to the text.
Additionally, we agree that pioneering studies on retromer from Burd lab needs proper citations in our study. We have included them in our revised manuscript (line 83-88).

8 What is BCS? I could find nothing substantive in the methods section to detail the treatments of the cells with copper or chelating agents etc.

Response: BCS stands for batho-cuproine disulphonate. It is a commonly used copper chelator. We have added details about copper and BCS treatment in the materials and method section (line:465- 468)

9. The introduction and the discussion are both too long and wordy and lack focus and should be revised accordingly so that they respectively frame the question and then assimilate the data into what is currently known about retromer and ATP7B recycling.

Response: We have restructured the introduction and the discussion. The modified parts are highlighted in yellow. It now looks much comprehensive and focused.

Reviewer 2 Advance Summary and Potential Significance to Field:

This manuscript analyzes of the role of retromer in the trafficking of copper-transporting ATPase ATP7B, whose mutations cause inherited disorder of copper metabolism known as Wilson disease.

Relocation of ATP7B from the Golgi to late endosomes and lysosomes is required for sequestration of excess and, hence, potentially toxic copper, while reduction in copper levels stimulates retrieval of ATP7B to the Golgi, where it metalates several copper- dependent proteins. Although anterograde trafficking of ATP7B was thoroughly dissected, the mechanisms driving retrieval of ATP7B to the Golgi remain poorly understood. Here the Authors employed a combination of biochemical and cell

biology approaches to demonstrate that VPS35 drives copper-dependent trafficking of ATP7B from late endo- lysosomal vesicles to the Golgi. Altogether, the main findings of the paper provide new important insights in understanding of ATP7B trafficking mechanisms and their possible implication in Wilson disease and other pathologies. Therefore, the manuscript might be of great interest to the readership of the Journal of Cell Science.

Reviewer 2 Comments for the Author:
In my view, in its current form the manuscript needs revision to address the below comments.

1. It remains unclear whether and how VPS35-mediate trafficking of ATP7B contributes to maintenance of copper homeostasis. Does VPS35 silencing affects (i) intracellular copper levels, (ii) tolerance of the cells to copper or (ii) activity of ATP7B? This issue is of fundamental importance for understanding of the mechanisms behind the regulation of copper balance in health and disease and requires additional experiments to be clarified. Tolerance to copper might be easily evaluated with live-dead staining, labelling of apoptotic cell/nuclei and MTT assay. ATP7B activity might be evaluated with tyrosinase/L- DOPA method, which was used by corresponding author in several publications.

Response: We sincerely thank the reviewer for the comment. We have performed the experiments that, I believe, improves the quality of our manuscript. They are as follows: (1) We have utilized inductively coupled plasma - optical emission spectrometry (ICP-OES) to measure copper in cells either treated with shRNA targeting VPS35 or scrambled shRNA (control) (Fig7B). We found lower copper levels in shVPS35 treated cells. (2) The data suggests that ATP7B arrested in the lysosomes are active and continue their copper export function, leading to lower intracellular copper. Due to the present pandemic situation, delivery of Menkes Fibroblast cells from US to India was not possible. (3) We have also measured cell viability using Neutral Red (NR) assay comparing cells treated with shRNA targeting VPS35 vs scrambled RNA. We did not notice and appreciable and statistically significant variation (Fig.7A). Further no overall change in the morphology was observed in cells treated with shRNA targeting VPS35 or scrambled shRNA (control) (Fig.S8B).

2. Discussion. Recent JCS publication (Stewart et al., 2019) shows that the retrograde ATP7B trafficking might be also regulated by COMMD1, whose silencing results in increased colocalization of ATP7B with VPS35. COMMD1, as a part of CCC complex, has been shown to cooperate with VPS35 in retrograde trafficking of ATP7A. I think that this should be discussed especially in the context of "large" protein complex that might bridge VPS35 to ATP7B.

Response: We thank the reviewer for the suggestion. Not only we have included this in the discussion, but also conducted experiments comparing retrograde trafficking of ATP7B in cells overexpressing GFP-wtCOMMD1 and two mutants (WD patient mutation, T174M and PtdIns(4,5)P2 interacting residues K167/173E) (ref Stewart et a 2019l). The constructs were kind gift from Dr. Jason Burkhead. We found that in cells overexpressing the mutant COMMD1, ATP7B failed to recycle back to the TGN in contrast to cells overexpressing the wtCOMMD1. We have included this data in text as a separate section in Results and in Fig.4Eii. This observation is in agreement with the study by Stewart et al that shows COMMD1 silencing results in increased colocalization of ATP7B and VPS35.

3. Discussion. The authors say that they never observed ATP7B at the plasma membrane of HepG2 cells (even treated with copper). It would be probably worth to specify that HepG2 cells were not polarized in this study, because in polarized HepG2 cells ATP7B is detectable at the apical surface.

Response: We agree with the reviewer that this might be an effect of non-polarized HepGs cells. We have specified this point in the text (line 389-390). To confirm, we costained ATP7B with phalloidin (staining primarily cortical actin) and Na,K-ATPase. We did not find any colocalization.

4. Page 8. The Authors say that they confirmed the role of retromer by rescuing the non-recycling phenotype of ATP7B in VPS35 kd cells by overexpressing wt-VPS35. I suggest testing

VPS35-R107A in rescue experiments. According my expectations, it should not rescue non-recycling phenotype of ATP7B or at least should not be as efficient as wt- VPS35.

Response: As per reviewer's suggestion, we have repeated the rescue experiment both with wt and the mutant (R107A) VPS35. The mutant could not rescue the non-recycling phenotype of ATP7B in VPS35 KD cells (Fig4D). This observation is in agreement with the experiment where we observe a drastically reduced colocalization and co-dwell time of ATP7B and mutant VPS35 (FigS6Ai,Aii & video 2A & B).

5. Western blots in figures 2D, 4A, 5A, and S2B lack input marker (GAPDH, actin or tubulin), which has to be provided.

Response: Fig 2D (revised as Fig.2E) As per reviewer's suggestions, we repeated the experiment to determine whether high copper leads to degradation of ATP7B in the lysosomes. We did not observe any significant degradation of ATP7B. We used GAPDH as the conventional control. Further we used two additional controls, Lamp1 as a lysosomal protein and Na,K-ATPase as a membrane protein. In both the cases we did not observe and significant reduction in their abundance in high copper.

Fig.4A (revised as Fig.3A). We repeated the experiment with control (ɣ-actin). We found VPS35 and VPS26 expressed in HepG2 cells.
Fig.5A (revised as Fig.4A). We ran the protein prep from cell pellets that previously showed VPS35 KD and probed it with housekeeping protein GAPDH. We found no change in GAPDH abundance.
Fig.S2B (revised as Fig.S5B). VPS26 KD was observed in cells where VPS35 was downregulated. Hence, the control (GAPDH) used in Fig.4A also serves as the control in this experiment. GAPDH, VPS35 and VPS26 were probed in the same set of cells.
To be sure of that knockdown of VPS35 was successful, we determined effect of KD on trafficking of M6PR that has been known to be regulated by the retromer complex (Fig.S5C) (Seaman, J Cell Biol, 2004 Volume 165, 111-122. We found that its recycling to TGN was abrogated in VPS35 KD cells.

Reviewer 3 Comments for the Author:

Title: Retromer retrieves the Wilson Disease protein, ATP7B from lysosomes in a copperdependent mode Comments to the author: Copper is a red-ox metal and acts as an essential micronutrient for all cells. Intracellularly, copper homeostasis is maintained through two different transporters, ATP7A and ATP7B, wherein ATP7A expresses ubiquitously and ATP7B is limited to liver, brain and kidney.

Mutations or defective function of these proteins results in Menkes disease and Wilson disease, respectively. Both proteins localize to Golgi (majorly to TGN) and supply the copper to the biosynthetic/secretory cuproproteins. ATP7A maintains copper levels by cycling between TGN and cell surface depending on its intracellular concentration. In contrast, ATP7B traffic to lysosomes from TGN at elevated copper levels; and the lysosomes dispose the copper to extracellularly through lysosomal exocytosis. However, it is not clear whether ATP7B can recycle back to TGN from lysosomes during the reversal of high to basal concentration of copper. Moreover, the mechanism behind this process is not well studied.

In this study, authors established that recycling of ATP7B from endolysosomes or LAMP-1-positive compartments to TGN requires retromer complex consisting VPS35, 26 and 29. Using high end imaging, UVcrosslinking and biochemical interaction studies, authors showed that ATP7B localize juxtaposed and interacts with VPS35 at high concentration of copper availability. Further, retromer knockdown experiments demonstrated that the complex is required for retrograde transport of ATP7B from LAMP-1-positive compartments to TGN. This study is quite unique and highlighted that the transporter ATP7B can recycle back to TGN through retromer for next round of copper transport.
However, authors should consider reviewing the below suggestions, which may improve their manuscript.

1. Authors indicated that ATP7B recycles from lysosomes in response to copper. However, it is not clear from their studies that the organelles are late endosomes or lysosomes or endolysosomes. In majority of the time authors used either Rab7 or LAMP1 as a marker; and indicated that those are lysosome (including the manuscript title). Authors should consider to use dextran uptake to distinct the terminal lysosomes vs endo-lysosome intermediates. Moreover, even in their model figure they did not indicated the nature of the organelles and the structures look like MVBs/recycling endosomes.

Response: We agree with the reviewer that the identity of the lysosomes harboring ATP7B should be established. As per reviewer's advice, we have labeled the lysosomes with Dextran using the well- established Dex-uptake assay. In parallel we have labeled the active hydrolase + compartments using the 'Magic red' assay (described in Bright et al., 2016, Current Biology 26, 2233–2245). We have determined the distribution of ATP7B among these two-type of compartments (Dextran-only and Dextran-Magic Red) in high copper condition. We have used two concentration of copper, i.e., 50 μM and 250 μM. We found ATP7B to localize in both these compartment types in both copper concentrations with a higher abundance in the acid hydrolase recycling compartments (Fig.2C, Fig.2D and Fig.SS4). Upon triggering the retrograde pathway in presence of cyclohexamide, we found ATP7B to return back to TGN under both 50 and 250 μM copper condition (text added in line: 137-162). We now have a clear idea about localization of ATP7B in 'active endolysosomes' vs 'inactive storage lysosomes' in high copper.
We have modified the model figure (Fig.8) as per the suggestion of the reviewer.

Also note that authors concluded that retromer sorts ATP7B from lysosomes and late endosomes (line 104); however, the title and abstract say only lysosomes. Earlier point is consistent with their SIM data that ATP7B localizes to late endo-lysosomal compartments (see line 141, Fig. 3).

Response: We have made appropriate changes as per reviewer's suggestions. We now have a better understanding of the compartments from which ATP7B is sorted back to the TGN. Using Dextran uptake and Magic Red assay we have established that ATP7B is primarily sorted from 'active hydrolase (cathepin B) positive endolysosomes (text added in line: 137-162). The title and the abstract has been modified. All changes are marked in yellow highlight.

2. In Figure 1A (panel 2), the Golgi (Golgin97 staining) appears to be dispersed upon treatment of cells with 50 μM for 2 h. Authors can explain the reason for this TGN/Golgi dispersal? Instead of overlaps plots (or in addition to these plots), authors should consider including the inset in the figure to emphasize the colocalization.*

Response: We apologize for lack of clarity in our presentation. It is reported as well as we have noticed that the structure of TGN (labeled by Golgin 97) is highly variable. It varies from a tight perinuclear staining to a looser vesicular staining and it stays unchanged with copper treatment. Coincidentally, the cell in image of Fig1A (panel 2) had a more dispersed Golgin 97 staining. This is not due to copper treatment.

As per reviewer's suggestion, we have added magnified field view (last image column) of colocalized/non-colocalized regions in Fig1.

3. Authors showed colocalization of ATP7B with LAMP1 (r=>0.5, equivalent to TGN, in Fig. 1B) and Rab7 (r=<0.1) post copper treatment (lines 120-121). Author should note that significant amount of LAMP1 also present in endocytic compartments including Golgi/associated vesicles. Thus, labeling the lysosomes with other markers such as LAMP-2 or Arl8b or labeled dextran (post uptake) will provide better conclusion to their studies.

Response: We thank the reviewer for this suggestion. We have labeled the lysosomes with Lamp2 (in addition to Lamp1) and dextran- Alexa Fluor-647. At high copper, ATP7B exhibits high colocalization coefficient with both these markers (Lamp2: Figs.S1 and DexA: Fig.2C &Fig.S4). Further, ATP7B traffics back from the Lamp2 positive vesicles upon copper removal.

We have labeled the lysosomes with Dextran using the well-established Dex-uptake assay. In parallel we have labeled the active hydrolase positive compartments using the 'Magic red' assay.

We have determined the distribution of ATP7B among these two-type of compartments (Dextran-only and Dextran-Magic Red) in high copper condition. We have used two concentration of copper, i.e., 50 μM and 250 μM. interestingly, we found ATP7B to localize in both these compartment types in both copper concentrations with a higher abundance on active lysosomes (Fig2C&D and FigS4). Upon triggering the retrograde pathway in presence of cyclohexamide, we found ATP7B to return back to TGN under both 50 and 250 μM copper condition. We now have a clear idea about localization of ATP7B in 'active' vs 'inactive' lysosomes in high copper (text added in line: 137-162).

In Fig. 2: 1st row-the localization of ATP7B (see 1st panel) appears to be slightly different than Fig. 1 (1st panel) post BCS treatment. 3rd row- large number of cells still showing colocalization with LAMP1 post rescue of copper treatment.

Response: I agree that ATP7B looks slightly more dispersed in figure 2. This is because, BCS treatment was carried out for 30 mins post ATP7B copper induced vesicularization. On treating with BCS for 2 hours we see a more complete return to TGN as in 5th panel in Fig.1A. We have modified the figure and the present one in a better representation of out inference.

For BCS condition in Fig.1 and Fig.2, we have noticed this minor variation among different experiments and the pattern also depends on the plan of the captured image. However, on a whole, ATP7B shows a much higher abundance in its juxta-nuclear location reminiscent of the Golgi network in BCS condition.

In Fig. S1A: Upon BCS treatment, the localization of ATP7B appears to be dispersed (supposed to be intact and should be in TGN? Moreover, Rab7 staining is very variable between the panel of Fig. S1A and in 3rd row the staining intensity is increased. Consistently, the ATP7B with Rab7 colocalization values are very low compared LAMP1. Similarly, Rab11 staining in the cells is variable and appears to be background staining. Authors should revisit this data either by using Rab7 constructs for anti- Rab7/labelled Transferrin uptake or anti-transferrin receptor antibody foranti-Rab11.

Response: We agree with the reviewer that Rab7 and Rab11 staining was not clean.

We apologize for the inadvertent mistake that we made in Fig.S1. We have now modified the figure (revision Fig.S2A). We modified our blocking and staining protocol for Rab7. It now shows much lesser background staining and higher specificity. We increased our cell counts and have included the number of cells considered to derive the data as 'n' in the bar charts.

For studying colocalization of ATP7B with Rab11, in the original version of the manuscript, we have transfected the cells with mCherry-Rab11 plasmid that lead to some non-specificity and aggregation of mCherry-Rab11 in the Golgi. For the revised version (Fig. S2C), we used Rab11 antibody that is specific and free of background staining. In FigS2, it is evident that ATP7B colocalizes with Rab7 but not with Rab11. We have provided the new set of data with both the endosomal markers in the modified Fig.S2.

4. Authors showed degradation of ATP7B post 250 μM copper treatment (line 123, Fig. 2D). This data requires some controls: (a) should use proteasomal (MG132) and lysosomal (bafilomycin or other) inhibitors during the experiment- this helps to know the major degradative route of ATP7B; (b) should considered use some internal protein control (GAPDH/tubulin/actin) instead of protein estimation for normalization; (c) should considered to test whether LAMP-1 will also change during the treatment conditions.

Response: We thank the reviewer for the suggestion. We apologize for the inadvertent error in our experiment. We performed the experiment in triplicate and normalized the data with control GAPDH. We did not record any statistically significant change in the abundance of ATP7B among the three conditions, copper chelated, 50μM and 250μM copper. Further we used two additional controls, Lamp1 as a lysosomal protein and Na,K-ATPase as a membrane protein. In both the cases we did not observe any significant reduction in their abundance in high copper. We have incorporated the changes in the text (line: 152-155) and the Fig.2E and Fig.2F.

5. Authors performed SIM studies to distinguish ATP7B localization with Rab7/LAMP1 compartments. Their studies indicated that 44.9% of ATP7B with Rab7-LAMP1-hybrid compartments; around 4% of ATP7B with either Rab7 or LAMP1. However, their error bars are bigger than this difference? Based on this, authors concluded that ATP7B largely recycle from non-degradative compartments (see line 138). Without any experimentation, authors cannot conclude those ATP7B-positive compartments are of non-degradative nature. To prove this, authors have to use reporter assays using DQ-BSA or magic red. Another possibility may be of "enlarged endosomes/endo-lysosomes" (small cohort of Rab7 and LAMP1 also present in them), consistent with their measured sizes (20-200 µM, diameter).

Response: We absolutely agree with the reviewer that the identity of the lysosomes harboring ATP7B needs to be established. As per reviewer's advice, we have labeled the lysosomes with Dextran using the well-established Dex-uptake assay. In parallel we have labeled the active hydrolase positive compartments using the 'Magic red' assay. We have determined the distribution of ATP7B among these two-type of compartments (Dextran-only and Dextran-Magic Red) in high copper condition. We have used two concentration of copper, i.e., 50 µM and 250 µM. We found

ATP7B to localize in both these compartment types in both copper concentrations with a higher abundance in the active hydrolase positive compartments (Fig.2C &Fig.2D). Upon triggering the retrograde pathway in presence of cyclohexamide, we found ATP7B to return back to TGN under both 50 and 250 µM copper condition. We now have a clear idea about localization of ATP7B in 'active' vs 'inactive' lysosomes in high copper.

6. Authors used VPS35 (WT/R107A)overexpression or its knockdown to study the role of retromer in regulating ATP7B recycling. As a positive control, have the authors validated the trafficking of M6PR in any of these conditions?

Response: As per reviewer's suggestion, we have now validated VPS35 knockdown by staining CI-M6PR in cells transfected with either siRNA targeted against VPS35 or scrambled RNA. We have noticed a fraction of M6PR retained in vesicles in VPS35 KD condition, thereby indicating its reduced recycling to the trans-Golgi network as against the scrambled RNA set where we observe complete localization between CI-M6PR and TGN46 (Fig.S5C). This data corroborates with the published findings by *Seaman J Cell Biol, Volume 165, 2004; 111-122.*

7. Model (Fig. 8): Authors are not explained/discussed their model in the text? In the model, authors are showing that ATP7B recycles from "ENDOSOME" (not lysosomes)?

Response: We have made changes in the model that now provides a better view of our findings.

8. For discussion: Any model/mechanisms that triggers the ATP7B recycling back to TGN from LEs/endolysosomes or how the high copper treatments hold the ATP7B protein on LEs/endolysosomes rather than its internalization to ILVs followingdegradation?

Response: We thank the reviewer for the suggestion. We have incorporated the changes in the discussion as suggested (line: 411-418).

9. In discussion: The luminal loops of ATP7B between the 8 TM domains are small and probably escapes lysosomal hydrolases (line 278). This hypothesis may not correct. Since, ATP7B may follow the same trafficking route similar to LAMP1 protein (or) ATP7B may not ubiquitinated/not sorted into ILVs by ESCRT machinery. However, authors observed that ATP7B degradation at 250 µM (2 h) treatment condition (line 293). This may be due to induced luminal changes in endosomes with high copper that probably lead to ATP7B sorting to ILVs following lysosomaldegradation.

Response: We thank the reviewer for the suggestion. We have incorporated the changes in the text as suggested. We apologize with our incorrect inference of ATP7B degradation at 250µM copper in our original version. We repeated the experiment with multiple proper controls. We did not observe any degadtion. We have modified the text accordingly (line: 155-156). This data is now incorporated as Fig.2E.

10. Authors showed high resolution deconvolved image of cells visualizing the colocalization between ATP7B, LAMP1 and VPS35 (see Fig. 7). In this image, authors should consider counting the number of these clusters in respect to LAMP1-positive organelles. This probably provide an additional information that the retromer dependent sorting of ATP7B possibly occurs from a cohort of Rab7- LAMP1-hybrid organelles.

Response: We thank the reviewer for the suggestion. We have incorporated the changes in the text (materials and method) as suggested. A total of 12 cells were taken to calculate ATP7B and VPS35 signals that are colocalizing together in Lamp1 positive compartments in high copper. 88 ATP7B (green) puncta were considered as actual signals. This served as reference to calculate relative abundance of green (ATP7B), cyan (VPS35), red (Lamp1) signals that are clustered together. 37 such triple coloured clusters were obtained (text modification in line: 650-655).

Minor comments:

1. Better running title: Retromer regulates retrograde trafficking of ATP7B

Response: We have incorporated the changes as suggested. It now reads: Retromer retrieves ATP7B from endolysosomes

2. Typos: trans Golgi network (line 37); P-type ATPases (line 38); remove Trans Golgi Network (line 46); exports copper into lysosomes (line 49); remove 1455 as superscript on AA (line 74); replace trans-Golgi network to TGN (line 108); Fig. 2, C and D - change it to Fig. 2, D and E (line 123); GFP (line 195); remove used as (duplicated in line 250); anti-VPS26 (line 261); and through lysosomes "in hepatocytes" (line 270);

Response: We have incorporated the changes as suggested.

3. Line 47: ceruloplasmin- has not been shown to localize to the lumen of TGN?

Response: We have incorporated the changes in the text as suggested (line 52).

4. Line 70: di/tri leucine motif - it should be dileucine/acid dileucine motif

Response: We have incorporated the changes in the text as suggested (line: 416).

5. Line 74: ATP7A to be important for its "retrieval" from cell membrane - 'endocytosis' is the better word

Response: We have incorporated the changes in the text as suggested.

6. Line 89: Authors should note that SNX17 has been shown to form a complex with CCC and WASH complexes, known as 'retriever', regulates retromer-independent endosomal cargo recycling to plasma membrane (PMID: 28892079)

Response: We have incorporated the changes in the text as suggested and removed SNX17 as per reviewer's advice.

7. Fig. 3: Authors should highlight the source of inset in the main panel and label the inset with respective fluorophore markers.

Response: We apologize for lack of clarity in explaining the figure. This is now Fig.S3A in the revised manuscript. We have incorporated the magnified view in the fig as suggested. The SIM image in Fig3B is a field from a region of a single cell and is not the same field as of Fig.3A. This image because of higher resolution provides a better clarity of colocalization of the three proteins.

8. Line 153: CI-M6PR recycles to TGN from "lysosomes" in a retromer regulated fashion. Note that both references clearly suggest that CI-M6PR recycles from "endosomes"

Response: We have incorporated the changes in the text as suggested (line: 169-170).

9. Line 166: Based on immunoblotting analysis, authors indicated that copper incubation does not alter abundance of VPS35 in HepG2 cells. Have the authors noticed any change in membrane recruitment of VPS35 during their conditions?

Response: We did not notice any alteration in localization of VPS35 in response to copper.

10. Fig. S2: (B) include some internal control to the blot (also to Fig. 4A, Fig. 5A); (C) Rab7- compartments are smaller than ATP7B punctate structures?

Response:

Fig.4A (revised as Fig.3A). We repeated the experiment with proper control (ɣ-actin). We found VPS35 and VPS26 expressed in HepG2 cells.
Fig.5A (revised as Fig.4A). We ran the protein prep from cell pellets that previously showed VPS35 KD and probed it with housekeeping protein GAPDH. We found no change in GAPDH abundance.
Fig.S2B (revised as Fig.S5B). Reduced expression of VPS26 was observed in cells where VPS35 was knocked down. Hence, the control (GAPDH) used in Fig.4A also serves as the control in this experiment. GAPDH, VPS35 and VPS26 were probed in the same set of cells.

11. Fig. 7: A-D Label the image with respective proteins. Similarly in Fig. S3

Response: We have made changes as suggested.

12. Have the authors used Ceruloplasmin as positive control in their GFP-ATP7B IP (Fig.S4)?

Response: We did not use Ceruloplasmin as a positive control. However, we have established the interaction between ATP7B with VPS35 with two independent experimental techniques, (A) Immunoprecipitation following in-vivo crosslinking and the Proximity Ligation Assay (Fig.6E &F). Further, we have functionally established that the retromer interacting Rab-GTPase, Rab7, upon downregulation also blocks the retrograde trafficking of ATP7B. These three evidences convincingly establishes the interaction between ATP7B and the retromer complex.

13. In Fig S1, authors indicated that Rab11 compartments as mid-endosomes. Better to label these organelles as recycling endosomes (REs).

Response: We agree that recycling endosome is more apt and have modified the sentence accordingly.

Second decision letter

MS ID#: JOCES/2020/246819

MS TITLE: Retromer retrieves the Wilson Disease protein ATP7B from endolysosomes in a copper-dependent mode

AUTHORS: Arnab Gupta, Santanu Das, Saptarshi Maji, Rutu Raj, Indira Bhattacharya, Tanusree Saha, and Nabanita Naskar

ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but reviewer #3 has two additional suggestions that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The authors have responded to the concerns I raised in my previous review and have made appropriate revisions to the manuscript. I can now recommend publication.

Comments for the author

The revisions have addressed the concerns I raised previously.

Reviewer 2

Advance summary and potential significance to field

Altogether, the main findings of the paper provide new important insights in understanding of ATP7B trafficking mechanisms and their possible implication in Wilson disease and other pathologies.

Comments for the author

The authors seriously addressed all my comments and significantly improved the manuscript over its previous version.

Reviewer 3

Advance summary and potential significance to field

The authors answered all my comments satisfactory. In this revision, the authors probed the ATP7B localization to endolysosomes (at higher copper condition) using substrates for lysosomes in addition to LAMP-1/2 markers. Moreover, the authors included the new data on COMMD1 to strengthen the role of retromer in recycling ATP7B from endolysosomes to TGN.

Comments for the author

I have noticed:
(1) Model figure: +Cu and -Cu was indicated for anterograde and retrograde transport- representation may not correct; may indicate high or low copper condition; and (2) Fig. 2Ci-left, bottom panel; appear that the image did not have any independent MR- positive compartments?

Second revision

Author response to reviewers' comments

Reviewer 3 Comments for the Author:
I have noticed:

Comment (1): *Model figure: +Cu and -Cu was indicated for anterograde and retrograde transport- representation may not correct; may indicate high or low copper condition*

Response: We agree with reviewer that 'high' and 'low' copper will be more appropriate. We have modified the figure as suggested.

Comment (2): *Fig. 2Ci-left, bottom panel; appear that the image did not have any independent MR- positive compartments?*

Response: DexA compartment depicts the total endolysosomal pool; DexA-MR compartment depicts active hydrolase endolysosomal pool and DexA only (MR negative) compartments depicts storage endolysosomal pool.

We agree that we did not notice independent Magic Red positive compartments in this field (shown in fig.2). But in other fields we noticed a very small number of MR-only compartment. Presence of MR- only compartment is probably a background or non-specific staining. Hence MR-only compartment are very few as also determined and measured statistically (falls with 5% error range) (depicted in Fig. 2D).

Third decision letter

MS ID#: JOCES/2020/246819

MS TITLE: Retromer retrieves the Wilson Disease protein ATP7B from endolysosomes in a copper-dependent mode

AUTHORS: Arnab Gupta, Santanu Das, Saptarshi Maji, Rutu Raj, Indira Bhattacharya, Tanusree Saha, and Nabanita Naskar
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.