EMBO reports

TMEM41B is a novel regulator of autophagy and lipid mobilization

Francesca Moretti, Phil Bergman, Stacie Dodgson, David Marcellin, Isabelle Claerr, Jonathan M. Goodwin, Rowena DeJesus, Zhao Kang, Christophe Antczak, Damien Begue, Debora Bonenfant, Alexandra Graff, Christel Genoud, John S. Reece-Hoyes, Carsten Russ, Zinger Yang, Gregory R. Hoffman, Matthias Mueller, Leon O. Murphy, Ramnik J. Xavier, Beat Nyfeler

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 6 March 2018

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting. However, all referees also point out that some of the conclusions require further data to substantiate them. Referee 2 and 3 are concerned that the knockdown of VMP1 appears to be incomplete. Referee 1 notes that more data are required to sustain the conclusion that TMEM14B regulates lipid droplets and mitochondria homeostasis and importantly, that it does so in a functional interaction with VMP1. Also the data on TMEM14Bs role in autophagosome biogenesis need to be strengthened. Referee 1 considers the data on mitochondrial homeostasis rather preliminary and referee 2 agreed with this evaluation upon further discussion.

Upon further discussion with the referees, we suggest to focus the study on autophagosoome biogenesis and contact sites. The data on mitochondrial homeostasis could either be strengthened or might be part of a future study. The data on the role of TMEM41B in autophagosome formation should be strengthened. Moreover, the putative functional interplay between TMEM41B and VMP1 at ER-mitochondria contact sites should be elucidated in more detail and substantiated.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

Supplementary/additional data: You can submit up to 5 images as Expanded View. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page with page numbers, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Regarding data quantification, please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where the requested information can be found.
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files in high resolution
(In order to avoid delays later in the publication process please check our figure guidelines before preparing the figures for your manuscript:
http://www.embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf)
- a separate PDF file of any Supplementary information (in its final format)
- all corresponding authors are required to provide an ORCID ID for their name. Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (http://embor.embopress.org/authorguide).

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

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

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REFEREE REPORTS

Referee #1:

Moretti and colleagues report the putative role of TMEM41B, an ER-protein identified via a pool CRISPR screening, in the autophagy pathway and its importance in lipid droplets and mitochondria homeostasis. Overall, the results sound interesting and the topic is of general interest for the cell and molecular biology community. However, many conclusions arise from over-interpretation of data, some of them being of poor quality. Importantly, there is an obvious lack of experimental justification all along the results section and my feeling is that the paper is constructed via an assembly of different kind of experiments, some of them being very interesting, but without strong link(s). My main criticism concerns the experimental setup used to study the implication of TMEM41B in autophagosome biogenesis sequence as well as the strong weakness of experiments aimed at investigate the function(s) of TMEM41B in lipid droplets and mitochondria "homeostasis" regulation. Finally, the experimental line-up of the article is often not clear, especially concerning the role of TMEM41B regarding lipid droplets and mitochondria biology.

MAJOR ISSUES:
- Some of the results shown in the figures 2 and 3 (and associated EV2) are puzzling: 1) it is quite difficult for readers to estimate/understand the data obtained by immunofluorescence microscopy (from figure 2 but also figure 3) when only merged pictures (all channels) are shown: authors must show each channel separated and illustrate more precisely the kind of phenomenon or phenotype (arrowheads, multi-color insets, etc.) they wish to describe. Moreover, especially in figure 2, the overall quality of imaging - like LC3 or calnexin signal - is not always very convincing. Finally, beside the fact that it is not clear to me why authors chose to show negative greyscale pictures in Fig2A and B, the conclusions described in the results section about the TMEM41B depletion putative effect on endosomes, ER and Golgi should be i) justified, ii) quantified somehow and iii) should be addressed as well by biochemical analyses.

As said in my general comment, many of the data presented in this paper are not enough justified from a biological point of view: for example, why the authors chose to look at markers of the secretory and endocytic pathway in cells depleted for TMEM41B?

The implication of TMEM41B in autophagosome biogenesis sequence is not convincing enough with the presented data: authors could benefit from other classical read-outs to clarify this point, such as time-point flux experiments (with LC3 tandem and Baf treatments), electron microscopy and analysis of other pre-autophagosomal markers, such as Beclin1, ATG14, DFCP1 and PI3P itself.

The manuscript is very weak concerning the conclusions about VMP1 and TMEM41B role in lipid droplets and mitochondria homeostasis. The link with VMP1 must be better introduced and protein-protein interaction must be investigated to sustain authors' conclusions. Moreover, the data concerning the re-localization of TMEM41B to Golgi are not clear, and not discussed. Analysis of lipid droplets behavior upon TMEM41B depletion is not enough explored: authors could investigate markers of the lipid droplets machinery (such as PLINs proteins) in terms of amount (by western blot for example) and intracellular distribution. Finally, based on the facts that "VMP1 regulates the dynamics of ER-mitochondria contact sites" (N.B: VMP1 is not only present at ER-mitochondria contact sites but also at ER-endosomes and ER-plasma membrane contact sites), authors analysed the mitochondrial network as well as mitochondrial respiration and conclude that "TMEM41B supports mitochondrial homeostasis"... what does that mean exactly? This must be discussed and justified.

Based on the above remarks and from the perspective of a TMEM41B novel function discovery, it would have been more useful, and beneficial for the paper's impact, to center the study on the putative presence (and connection/dependency) of TMEM41B to VMP1-associated contact sites rather than looking (rapidly) to organelle(s) "homeostasis".

MINOR COMMENTS:
1) in the introduction, authors state that autophagosome biogenesis "requires the interplay of various

cellular organelles and compartments [...]" and refer as well to ER-mitochondria contact sites; it is noteworthy that also ER-lipid droplets and ER-plasma membrane contact sites have been shown to participate in autophagy regulation as well.
2) it is not clearly stated why the authors have based their elegant CRISP pool screening, and autophagy readouts, on cargoes degradation (p62 and TAX1BP1, and latter Salmonella bug): it is well known that non-selective autophagy also exists and that cargoes behavior could not account for all autophagy and thus autophagosomes formation.
3) why authors used H4 cells? What is the biological relevance?
4) authors used AZD8055 mTOR related drug to induce autophagy: how is it justified? The study could strongly benefit from more widely used autophagy associated stimuli, such as starvation (kinetics).
5) it is not very clear why authors are showing results using the anti-TMEM41B antibody (Fig 3A): what is the benefit to show an antibody which is not recognizing endogenous protein in the study?
6) the presence of overexpressed TMEM41B at ER should be documented as well with colocalization with bona fide marker of ER membrane, such as Sec61beta(GFP or RFP), since the staining obtained with anti-CANX is not fully covering the ER (as expected).
7) results shown in the figure EV3 could benefit to be quantified for the LD volume (in µm3) rather than "size" in µm².
8) the Figure 4F is not sustained by the current results, because the data are too preliminary.

Referee #2:

The paper by Moretti et al entitled "TMEM41B is a novel transmembrane protein required for autophagy initiation and organelle homeostasis" describes the use of a FACS-based pooled CRISPR screening approach to identify the 6 TM transmembrane protein TMEM41B as a novel ER-resident regulator of autophagy whose knockout (KO) phenotype negatively affects autophagosome formation. The authors put forward a model where the autophagosomes form but their completion is inhibited in TMEM41B KO or knockdown (KD) cells.
The paper is concise and very well written. The novelty aspect is obvious since a new autophagy regulator at the ER membrane is presented that is needed for efficient completion of autophagosome formation. The data presented are for the most part convincing with relevant controls. However, more of the data need to be quantified to substantiate the conclusions made.
1. Fig. 1 D-E: The blots of p62 and NDP52 should be quantified.
2. LC3B punctae formation in Fig 2A need to be quantified, as do the intensities of NDP52 and p62 staining.
3. On page 5 line 6 from the top the statement "Deletion of TMEM41B does not appear to cause a late stage block in autophagy as p62 and NDP52 did not accumulate in endolysosomal puncta." is not substantiated in any data shown as far as I could see. The authors need to show these data.
4. The imaging data in Fig. 2D need to be quantified to show the degree of colocalization of LC3B and WIPI2 by for example using Manders colocalization coefficient.
5. In Fig. 3C the knockdown (KD) of VMP1 is not very impressive and the effects of KD of TMEM41B and of VMP1 on the levels of p62 and LC3-II not very prominent either and quantifications are missing.
6. A weakness is of course that the lack of an antibody efficiently recognizing the endogenous protein precludes monitoring endogenous levels and studies of the localization of the endogenous protein. However, it may often be very difficult to obtain antibodies towards small transmembrane proteins where only small portions of the protein are exposed to the cytosol. This is therefore not a requirement for this study and should be regarded as a challenge for future studies of TMEM41B.

Referee #3:

In the manuscript by Moretti et al., the authors identify a role for TMEM41B in autophagy. Using a CRISPR screen based on GFP-p62 and NDP52, the authors find that loss of TMEM41B impairs their turnover and go on to show that autophagy appears arrested at an early stage and salmonella replication, which can be reduced by autophagy, is increased. The authors go on to show that exogenous TMEM41B localises to the ER and interacts in the same pathway as VMP1, an ER-resident protein previously implicated in autophagy. Finally, the authors show that loss of TMEM41B disrupts lipid droplets as well as the mitochondrial network and OXPHOS, again in a

similar manner to loss of VMP1, further strengthening the notion that these two proteins operate in the same pathway.
This is an intriguing manuscript and although there is not an in-depth characterization of TMEM41B, it does assign a new role for TMEM41B in autophagy and as such will be of interest to a wide audience. I do have some points that require clarification though.

1) I was a little confused about how the authors determined that they had knocked out TMEM41B. They state that they could not identify an antibody to detect endogenous protein, but that "an editing frequency of 63% was confirmed for the TMEM41B genomic locus". What does this mean? Are the authors working with a complete knockout of all alleles? Are they working with a single clone and what are the genomic mutations? Can the authors use RT-PCR to confirm KO?
2) The differential effect of TMEM41B of p62 and NDP52 under basal conditions, as highlighted in Figure 1D, is very interesting. In most conditions I can think of, impairment of autophagy leads to increased p62 levels - even under basal conditions. Yet this is not occurring here. At the end of the Discussion, the authors mention that TMEM41B does not completely block autophagic flux and this might help explain why p62 does not accumulate, but I cannot find these data in the manuscript. The authors should perform the experiment shown in Figure 1D with/without bafilomycin to show the flux of p62/NDP52/LC3 under basal and induced conditions.
3) In Figure 3, the authors use exogenous cDNA for TMEM41B to determine its cellular localisation. While this is not a problem in itself, it can be artefactual if overexpression compared to endogenous levels is significant. Given that this is the first major characterization of this protein, it would be very informative and greatly strengthen the authors conclusions to have some endogenous data. I do appreciate that the authors have been unable to find an antibody to recognize endogenous TMEM41B, but it should be straightforward to use CRISPR/Cas9 to knockin a tag to enable endogenous protein detection.
4) In Figure 3C, the authors use sgRNA against VMP1, yet it does not look like it has resulted in knockout of the protein. Is this the case? If so it might be hard to reconcile the data comparing loss of TMEM41B with loss of VMP1 (if there is no loss of VMP1).

Minor points:
1) Many of the micrographs require scale bars and the immunoblots need MW markers.

1st Revision - authors' response 6 June 2018

Referee#1:

Moretti and colleagues report the putative role of TMEM41B, an ER-protein identified via a pool CRISPR screening, in the autophagy pathway and its importance in lipid droplets and mitochondria homeostasis. Overall, the results sound interesting and the topic is of general interest for the cell and molecular biology community. However, many conclusions arise from over-interpretation of data, some of them being of poor quality. Importantly, there is an obvious lack of experimental justification all along the results section and my feeling is that the paper is constructed via an assembly of different kind of experiments, some of them being very interesting, but without strong link(s). My main criticism concerns the experimental setup used to study the implication of TMEM41B in autophagosome biogenesis sequence as well as the strong weakness of experiments aimed at investigate the function(s) of TMEM41B in lipid droplets and mitochondria "homeostasis" regulation. Finally, the experimental line-up of the article is often not clear, especially concerning the role of TMEM41B regarding lipid droplets and mitochondria biology.

Referee #1 agrees that our results are interesting but points out that experimental justification and line-up is often not clear. In addition to strengthening the role of TMEM41B in autophagosome biogenesis, we are now including two additional datasets (screening of fluorescent probes, TMEM41B interaction proteomics) to clarify how we discovered a role for TMEM41B in lipid droplet regulation and support its putative function at ER contact sites. In addition, we are no longer focusing on general organelle homeostasis but added new data to document the role of TMEM41B in regulating lipid mobilization and β -oxidation.

MAJOR ISSUES:

- Some of the results shown in the figures 2 and 3 (and associated EV2) are puzzling: 1) it is quite difficult for readers to estimate/understand the data obtained by immunofluorescence microscopy (from figure 2 but also figure 3) when only merged pictures (all channels) are shown: authors must show each channel separated and illustrate more precisely the kind of phenomenon or phenotype (arrowheads, multi-color insets, etc.) they wish to describe.

We are now showing separate channels for merged pictures (Fig. 2E, 2H, 2J, 4A, 5A, 5B, EV3B, EV4A, EV4B, EV5B) and use arrowheads to point to our described phenotypes (Fig. 1G, 2E, 2H, EV5B).

Moreover, especially in figure 2, the overall quality of imaging - like LC3 or calnexin signal - is not always very convincing.

We have included quantifications of LC3 puncta (Fig. 2D) and added confocal images co-localizing LC3 puncta with WIPI2 as well as RFP-DFCP1 (Fig. 2E and 2H) to strengthen our data describing a role of TMEM41B in autophagosome biogenesis.

Finally, beside the fact that it is not clear to me why authors chose to show negative greyscale pictures in Fig2A and B, the conclusions described in the results section about the TMEM41B depletion putative effect on endosomes, ER and Golgi should be i) justified, ii) quantified somehow and iii) should be addressed as well by biochemical analyses.

As said in my general comment, many of the data presented in this paper are not enough justified from a biological point of view: for example, why the authors chose to look at markers of the secretory and endocytic pathway in cells depleted for TMEM41B?

We believe that the contrast of greyscale images is better suited to highlight cellular phenotypes, including puncta, and have kept this setting when showing separate channels for merged pictures. Our initial aim for including negative data on endosome, ER and Golgi morphology was to demonstrate that TMEM41B-depletion does not cause a general and pleiotropic effect on the secretory pathway. As we are not aware of biochemical assays to analyze endosomes, ER or Golgi morphology, we have decided to remove this data and highlight the specificity of the TMEM41B-depletion phenotype using the analysis of different fluorescent probes (Fig. 3 and EV3A).

The implication of TMEM41B in autophagosome biogenesis sequence is not convincing enough with the presented data: authors could benefit from other classical read-outs to clarify this point, such as time-point flux experiments (with LC3 tandem and Baf treatments), electron microscopy and analysis of other pre-autophagosomal markers, such as Beclin1, ATG14, DFCP1 and PI3P itself.

We have strengthened the role of TMEM41B in autophagosome biogenesis as follows:

1. *Protein levels of p62 and NDP52 were assessed in the absence or presence of Bafilomycin A1 (Fig. 1D and 1F). This data shows impaired lysosomal flux of p62 and NDP52 in TMEM41B KO cells consistent with our pooled CRISPR screening data.*

2. *In addition to WIPI2, we have analyzed DFCP1 as an additional pre-autophagosomal marker (Fig. 2H) as suggested by the reviewer. For both WIPI2 and DFCP1 we see enhanced co-localization with LC3 in TMEM41B KO cells (Fig. 2F and 2I).*

3. *We analyzed mCherry-GFP-LC3 in the absence or presence of Bafilomycin A1 (Fig. 2J). This data shows a significant decrease of mCherry- and GFP-positive LC3 puncta when TMEM41B KO cells were treated with bafilomycinA1, in line with impaired formation of autophagosomes.*

4. *We have performed qualitative ultrastructural analysis of TMEM41B KO cells by transmission electron microscopy (Fig. EV2). In addition to enlarged lipid droplets, we have identified an accumulation of electron dense structures upon TMEM41B depletion.*

Immuno-EM studies will be required for the precise characterization of these structures, which was not feasible in the timeframe of the revision. We suggest that the electron dense structure may represent isolation membranes accumulating as a result of stalled autophagosomes, consistent with what has been observed for VMP1-deficient cells [1, 2].

5. *We have included a reference to the work of Shoemaker et al. [3] that independently identified TMEM41B as a novel regulator of autophagy and showed that accumulated LC3-II remained largely trypsin-sensitive in a protease protection assay of TMEM41B KO cell extracts.*

The manuscript is very weak concerning the conclusions about VMP1 and TMEM41B role in lipid droplets and mitochondria homeostasis. The link with VMP1 must be better introduced and protein-protein interaction must be investigated to sustain authors' conclusions. Moreover, the data concerning the re-localization of TMEM41B to Golgi are not clear, and not discussed.

We have performed additional experiments to evaluate the link between VMP1 and TMEM41B:

1. *We have tested if TMEM41B overexpression can rescue the lipid droplet phenotype in VMP1 KO cells. While TMEM41B expression rescued lipid droplet size in TMEM41B KO cells, we did not see any rescue in VMP1 KO cells and conclude that TMEM41B cannot compensate for VMP1-deficiency (Fig. EV5B and EV5C).*

2. *We have performed interaction proteomics to identify TMEM41B binding partners (Fig. 5E, Dataset EV2). In this analysis, we failed to detect VMP1. In addition to the 1% Triton-X cell lysis buffer used for interaction proteomics, we have tested 2 additional lysis buffers (0.3% CHAPS and RIPA) but were unable to detect an interaction between TMEM41B and VMP1 by co-IP. We cannot exclude that we have not yet found the optimal lysis conditions to preserve a putative interaction between TMEM41B and VMP1.*

3. *In contrast to C-terminally Myc-tagged TMEM41B, we do not observe relocalization of untagged or N-terminally-tagged TMEM41B to the Golgi in the absence of VMP1. We believe that the Golgi re-localization of Myc-TMEM41B is due to the combined loss of two ER retention signals: 1) loss of the C-terminal COPI ER retention motif (discussed in the manuscript), 2) a VMP1-dependent ER retention (currently unclear if this is a direct or indirect effect since we were unable to detect an interaction between TMEM41B and VMP1 by co-IP).*

In light of these new results, we agree that our data on VMP1 are too weak and have decided to deemphasize the TMEM41B-VMP1 link in the revised manuscript. We now only describe the similarity of the autophagy and lipid droplet defects in TMEM41B and VMP1 KO cells (Fig. EV5). Instead, we have included a new TMEM41B interaction proteomics dataset and focus on SIGMAR1 which co-IPs with TMEM41B. SIGMAR is a well-characterized ER chaperone which regulates calcium and lipid transfer at the interface of the ER, lipid droplets and mitochondria, thereby strengthening the model of TMEM41B's role at ER contact sites.

Analysis of lipid droplets behavior upon TMEM41B depletion is not enough explored: authors could investigate markers of the lipid droplets machinery (such as PLINs proteins) in terms of amount (by western blot for example) and intracellular distribution.

We have further characterized lipid droplets in TMEM41B-depleted cells as follows:

1. We have analyzed additional fluorescent lipid probes (BODIPY 493, NBD cholesterol, BODIPY FL C12, NBD C6 ceramide, BODIPY FL C12 sphingomyelin) and show that neutral lipid and cholesterol probes specifically accumulate in droplets in TMEM41B KO cells (Fig. 3A and EV3A).

2. As suggested by the reviewer, we have analyzed the lipid droplet marker ADRP (also known as perilipin-2). Immunostaining in TMEM41B KO cells showed enlarged ADRP-labeled droplets (Fig. 3D and 3E), which co-localized with neutral lipids (Fig. EV3B). Western blot analysis showed statistically significant higher ADRP protein levels upon TMEM41B KO (Fig. 3F and 3G).

3. Ultrastructural analysis by transmission electron microscopy identified enlarged lipid droplets in TMEM41B KO cells (Fig. EV2).

All these data support our conclusions that TMEM41B-deficiency results in enlarged lipid droplets.

Finally, based on the facts that "VMP1 regulates the dynamics of ER-mitochondria contact sites" (N.B: VMP1 is not only present at ER-mitochondria contact sites but also at ER-endosomes and ER-plasma membrane contact sites), authors analysed the mitochondrial network as well as mitochondrial respiration and conclude that "TMEM41B supports mitochondrial homeostasis"... what does that mean exactly? This must be discussed and justified.

We have added new data to more specifically characterize the role of TMEM41B in regulating lipid mobilization and mitochondrial β-oxidation and have deemphasized a more general role of TMEM41B in organelle homeostasis.

1. *We have performed pulse and chase experiments to assess the mobilization of fluorescently labeled fatty acids from lipid droplets to mitochondria upon serum deprivation. Transfer of fatty acids to mitochondria was significantly impaired in TMEM41B KO cells (Fig. 4A and 4B).*

2. *In the first version of the manuscript, we have shown decreased mitochondrial respiration, which was paralleled by increased glycolysis in TMEM41B KO cells (now Fig. 4C). In the revision, we have now added Seahorse results for oxidation of endogenous fatty acids. For this purpose, cells were tested in substrate-limited medium (to sensitize for utilization of endogenous fatty acids) in the absence or presence of etomoxir, an inhibitor of carnitine palmitoyltransferase-1, which is essential for the transport of fatty acids into mitochondria. This experiment showed that the difference in mitochondrial respiration between TMEM41B KO and control cells was fully blunted with etomoxir (Fig. 4D and 4E). We conclude that impaired mitochondrial oxidative respiration in TMEM41B KO cells is likely due to defective utilization of endogenous fatty acids.*

Based on the above remarks and from the perspective of a TMEM41B novel function discovery, it would have been more useful, and beneficial for the paper's impact, to center the study on the putative presence (and connection/dependency) of TMEM41B to VMP1-associated contact sites rather than looking (rapidly) to organelle(s) "homeostasis".

As discussed above, we have deemphasized a more general role of TMEM41B in organelle homeostasis as well as the TMEM41B-VMP1 link in the revised manuscript. By including the TMEM41B interaction proteomics data and validating the TMEM41B-SIGMAR1 interaction by co-IP (Fig. 5E and 5F, Dataset EV2), we provide new data strengthening our model of TMEM41B regulating lipids at ER contact sites.

MINOR COMMENTS:

1) in the introduction, authors state that autophagosome biogenesis "requires the interplay of various cellular organelles and compartments [...]" and refer as well to ER-mitochondria contact sites; it is noteworthy that also ER-lipid droplets and ER-plasma membrane contact sites have been shown to participate in autophagy regulation as well.

We have updated the references to include ER-lipid droplets and ER-plasma membrane contact sites.

2) it is not clearly stated why the authors have based their elegant CRISP pool screening, and autophagy readouts, on cargoes degradation (p62 and TAX1BP1, and latter Salmonella bug): it is well known that non-selective autophagy also exists and that cargoes behavior could not account for all autophagy and thus autophagosomes formation.

We fully agree that we are not reading out all autophagy and autophagosome formation with p62 and NDP52 autophagy cargo receptor screens. However, work by Larsen et al. [4] showed that autophagy cargo receptors can be monitored as a proxy for the cellular autophagy capacity. Our

previous work also shows that autophagy cargo receptors assays can be exploited for forward genetic screening paradigms to robustly map the mammalian autophagy machinery [5] and we have clarified this in the introduction.

3) why authors used H4 cells? What is the biological relevance?

Lipinski et al used H4 cells for one of the first genomewide autophagy screens [6]. We have picked this cell line also based on technical feasibility studies (H4 cells divide, infect and edit well and are amenable to FACS sorting) and validated the line in our first pooled CRISPR autophagy screen [5].

4) authors used AZD8055 mTOR related drug to induce autophagy: how is it justified? The study could strongly benefit from more widely used autophagy associated stimuli, such as starvation (kinetics).

ATP-competitive mTOR inhibitors such as AZD8055 or Torin1 have been shown to robustly activate autophagy [7-10] and we have seen less cytotoxicity than upon starvation.

5) it is not very clear why authors are showing results using the anti-TMEM41B antibody (Fig 3A): what is the benefit to show an antibody which is not recognizing endogenous protein in the study?

We believe that it is important to rule out that the Myc-tag changes the function or localization of TMEM41B. For this purpose, we are using the anti-TMEM41B antibody to evaluate an overexpressed but untagged TMEM41B cDNA (Fig. EV1C and EV4A) and this is now clarified in the text.

6) the presence of overexpressed TMEM41B at ER should be documented as well with colocalization with bona fide marker of ER membrane, such as Sec61beta(GFP or RFP), since the staining obtained with anti-CANX is not fully covering the ER (as expected).

To further support ER localization of TMEM41B, we have now included co-localization with anti-KDEL (Fig. EV4B).

7) results shown in the figure EV3 could benefit to be quantified for the LD volume (in μm3) rather than "size" in μm².

We are acquiring images in single confocal planes and are not applying any 3D reconstruction. We assume that LDs are not perfect spheres and therefore prefer to analyze our images by quantifying droplets' area.

8) the Figure 4F is not sustained by the current results, because the data are too preliminary.

We now present a revised model in the Synopsis.

Referee #2:

The paper by Moretti et al entitled "TMEM41B is a novel transmembrane protein required for autophagy initiation and organelle homeostasis" describes the use of a FACS-based pooled CRISPR screening approach to identify the 6 TM transmembrane protein TMEM41B as a novel ER-resident regulator of autophagy whose knockout (KO) phenotype negatively affects autophagosome formation. The authors put forward a model where the autophagosomes form but their completion is inhibited in TMEM41B KO or knockdown (KD) cells.
The paper is concise and very well written. The novelty aspect is obvious since a new autophagy regulator at the ER membrane is presented that is needed for efficient completion of autophagosome formation. The data presented are for the most part convincing with relevant controls. However, more of the data need to be quantified to substantiate the conclusions made.

We thank Referee #2 for the appraisal of our manuscript. We have addressed the concerns around better quantification of the phenotypes as described below.

1. Fig. 1 D-E: The blots of p62 and NDP52 should be quantified.

Quantification of these western blots is now presented in Fig. 1E and 1F.

2. LC3B punctae formation in Fig 2A need to be quantified, as do the intensities of NDP52 and p62 staining.

Quantification of LC3B puncta is now shown in Fig. 2D, while quantifications of NDP52 and p62 stainings is shown in Fig. EV1A.

3. On page 5 line 6 from the top the statement "Deletion of TMEM41B does not appear to cause a late stage block in autophagy as p62 and NDP52 did not accumulate in endolysosomal puncta." is not substantiated in any data shown as far as I could see. The authors need to show these data.

We have removed this statement. The data presented in Fig. 1G and 1H show that p62 accumulates outside of LAMP1-positive organelles in TMEM41B KO cells, in support of our hypothesis that TMEM41B-depletion does not result in a late stage block in autophagy. This notion is also substantiated by a set of new data shown in Fig. 1D, 2H and 2J.

4. The imaging data in Fig. 2D need to be quantified to show the degree of colocalization of LC3B and WIPI2 by for example using Manders colocalization coefficient.

Colocalization was assessed for Fig. 2E and 2H using the COMDET plugin in ImageJ (https://imagej.net/Spots_colocalization (ComDet)) that is specifically suited to detect spot colocalizations. The results are expressed as % of WIPI2 or DFCP1 puncta positive for LC3 (Fig. 2F and 2I).

5. In Fig. 3C the knockdown (KD) of VMP1 is not very impressive and the effects of KD of TMEM41B and of VMP1 on the levels of p62 and LC3-II not very prominent either and quantifications are missing.

To address this point, we have isolated clonal KO lines for both TMEM41B and VMP1 and analyzed them side by side for autophagy and lipid droplets size (Fig. EV5). VMP1 KO in the selected clone is complete (Fig. EV5A) and TMEM41B alleles appear edited in the selected clone according to TIDE assay (see response to Referee #3 point 1). Both immunoblot data (Fig. EV5A) and lipid droplet images (Fig. EV5B) have been quantified.

6. A weakness is of course that the lack of an antibody efficiently recognizing the endogenous protein precludes monitoring endogenous levels and studies of the localization of the endogenous protein. However, it may often be very difficult to obtain antibodies towards small transmembrane proteins where only small portions of the protein are exposed to the cytosol. This is therefore not a requirement for this study and should be regarded as a challenge for future studies of TMEM41B.

We have now partially addressed this concern by showing ER localization for TMEM41B endogenously tagged with Myc (Fig. 5B-D). This was achieved by using CRISPR-mediated knock-in as described in response to Referee #3 point 3.

Referee #3:

In the manuscript by Moretti et al., the authors identify a role for TMEM41B in autophagy. Using a CRISPR screen based on GFP-p62 and NDP52, the authors find that loss of TMEM41B impairs their turnover and go on to show that autophagy appears arrested at an early stage and salmonella replication, which can be reduced by autophagy, is increased. The authors go on to show that exogenous TMEM41B localizes to the ER and interacts in the same pathway as VMP1, an ER-resident protein previously implicated in autophagy. Finally, the authors show that loss of TMEM41B disrupts lipid droplets as well as the mitochondrial network and OXPHOS, again in a similar manner to loss of VMP1, further strengthening the notion that these two proteins operate in the same pathway.

This is an intriguing manuscript and although there is not an in-depth characterization of TMEM41B, it does assign a new role for TMEM41B in autophagy and as such will be of interest to a wide audience. I do have some points that require clarification though.

We thank Referee #3 for the appraisal of our manuscript and have addressed the concerns as described below.

1) I was a little confused about how the authors determined that they had knocked out TMEM41B. They state that they could not identify an antibody to detect endogenous protein, but that "an editing frequency of 63% was confirmed for the TMEM41B genomic locus". What does this mean? Are the authors working with a complete knockout of all alleles? Are they working with a single clone and what are the genomic mutations? Can the authors use RT-PCR to confirm KO?

Since anti-TMEM41B did not detect the endogenous protein, we assessed the knockout by sequencing of the TMEM41B genomic locus. For this purpose, we used TIDE (Tracking of Indels by Decomposition) which consists of Sanger sequencing of two PCR products (one from wild type cells, one from KO cells) amplifying the region surrounding the sgRNA cutting site. The sequencing traces are then decomposed by an algorithm to quantify the percentage of edited sequences [11]. We believe that the sequencing-based TIDE method is more quantitative than RT-PCR-based approaches. We did most of our validation studies in a population of H4 Cas9 cells stably expressing the TMEM41B sgRNA and the TIDE method detected insertions and deletions in 63% of all sequences. We also analyzed clonal TMEM41B KO cells where the TIDE method detected insertions and deletions in 83% of all sequences with two specific editing events (2bp and 4bp deletion). As mentioned in the original publication, the TIDE tool is hampered by highly repetitive sequences which is the case for the sequences surrounding the TMEM41B sgRNA cutting site. We therefore believe that the TIDE method for TMEM41B is rather underrepresenting the editing frequency.

2) The differential effect of TMEM41B of p62 and NDP52 under basal conditions, as highlighted in Figure 1D, is very interesting. In most conditions I can think of, impairment of autophagy leads to increased p62 levels - even under basal conditions. Yet this is not occurring here. At the end of the Discussion, the authors mention that TMEM41B does not completely block autophagic flux and this might help explain why p62 does not accumulate, but I cannot find these data in the manuscript. The authors should perform the experiment shown in Figure 1D with/without bafilomycin to show the flux of p62/NDP52/LC3 under basal and induced conditions.

We have now included autophagy flux experiments:

1. *p62 and NDP52 immunoblots and their quantification in the absence or presence of Bafilomycin A1 are shown in Fig. 1D and 1F for both basal conditions (DMSO) as well as upon autophagy activation (AZD8055). These results are consistent with our screening data and show a significant difference in lysosomal flux for p62 in TMEM41B KO versus control cells only when autophagy was activated with AZD8055. For NDP52, we see a significant difference both basally or when autophagy was activated (Fig. 1F).*

2. *For LC3, we analyzed mCherry-GFP-LC3 in the absence or presence of Bafilomycin A1 (Fig. 2J). This data show a significant decrease of mCherry- and GFP-positive LC3 puncta when TMEM41B KO cells were treated with bafilomycinA1, in line with impaired formation of autophagosomes.*

3) In Figure 3, the authors use exogenous cDNA for TMEM41B to determine its cellular localisation. While this is not a problem in itself, it can be artefactual if overexpression compared to endogenous levels is significant. Given that this is the first major characterization of this protein, it would be very informative and greatly strengthen the authors conclusions to have some endogenous data. I do appreciate that the authors have been unable to find an antibody to recognize endogenous TMEM41B, but it should be straightforward to use CRISPR/Cas9 to knockin a tag to enable endogenous protein detection.

To address this point, we knocked-in a C-terminal Myc tag into the endogenous TMEM41B locus in HeLa cells (Fig. 5B-D) using CRISPR-mediated homologous recombination. Co-localization studies confirmed an ER localization for endogenous TMEM41B (Fig. 5B).

4) In Figure 3C, the authors use sgRNA against VMP1, yet it does not look like it has resulted in knockout of the protein. Is this the case? If so it might be hard to reconcile the data comparing loss of TMEM41B with loss of VMP1 (if there is no loss of VMP1).

See response to Referee #2 point 5.

Minor points:
1) Many of the micrographs require scale bars and the immunoblots need MW markers.

Missing scale bars have been added and MW markers for immunoblot micrographs can be found in the source data file.

1. Tabara LC, Escalante R (2016) VMP1 Establishes ER-Microdomains that Regulate Membrane Contact Sites and Autophagy. *PLoS One* **11**: e0166499
2. Calvo-Garrido J, Carilla-Latorre S, Lazaro-Dieguez F, Egea G, Escalante R (2008) Vacuole membrane protein 1 is an endoplasmic reticulum protein required for organelle biogenesis, protein secretion, and development. *Mol Biol Cell* **19**: 3442-53
3. Schoemaker CJ HT, Weir NR, Polyakov N, Denic V (2017) A CRISPR screening approach for identifying novel autophagy-related factors and cytoplasm-to-lysosome trafficking routes. In bioRxiv:
4. Larsen KB, Lamark T, Overvatn A, Harneshaug I, Johansen T, Bjorkoy G (2010) A reporter cell system to monitor autophagy based on p62/SQSTM1. *Autophagy* **6**: 784-93
5. DeJesus R, Moretti F, McAllister G, Wang Z, Bergman P, Liu S, Frias E, Alford J, Reece-Hoyes JS, Lindeman A*, et al.* (2016) Functional CRISPR screening identifies the ufmylation pathway as a regulator of SQSTM1/p62. *Elife* **5**
6. Lipinski MM, Hoffman G, Ng A, Zhou W, Py BF, Hsu E, Liu X, Eisenberg J, Liu J, Blenis J*, et al.* (2010) A genome-wide siRNA screen reveals multiple mTORC1 independent signaling pathways regulating autophagy under normal nutritional conditions. *Dev Cell* **18**: 1041-52
7. Thoreen CC, Kang SA, Chang JW, Liu Q, Zhang J, Gao Y, Reichling LJ, Sim T, Sabatini DM, Gray NS (2009) An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. *J Biol Chem* **284**: 8023-32
8. Nyfeler B, Bergman P, Triantafellow E, Wilson CJ, Zhu Y, Radetich B, Finan PM, Klionsky DJ, Murphy LO (2011) Relieving autophagy and 4EBP1 from rapamycin resistance. *Mol Cell Biol* **31**: 2867-76
9. Chresta CM, Davies BR, Hickson I, Harding T, Cosulich S, Critchlow SE, Vincent JP, Ellston R, Jones D, Sini P*, et al.* (2010) AZD8055 is a potent, selective, and orally bioavailable ATP-competitive mammalian target of rapamycin kinase inhibitor with in vitro and in vivo antitumor activity. *Cancer Res* **70**: 288-98
10. Huang S, Yang ZJ, Yu C, Sinicrope FA (2011) Inhibition of mTOR kinase by AZD8055 can antagonize chemotherapy-induced cell death through autophagy induction and down-regulation of p62/sequestosome 1. *J Biol Chem* **286**: 40002-12
11. Brinkman EK, Chen T, Amendola M, van Steensel B (2014) Easy quantitative assessment of genome editing by sequence trace decomposition. *Nucleic Acids Res* **42**: e168

2nd Editorial Decision 23 June 2018

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, all referees appreciate that the data on the proposed role of TMEM41B in autophagy have been considerably strengthened during the revision. However, referee 1 and 3 both note that the proposed links to LD metabolism and in particular the functional link to SIGMAR1 remain rather preliminary and speculative at this point and that more rigorous experiments would be

required before publication. In this context, referee 3 suggested to remove the data on the SIGMAR1 interaction and save it for a future, more detailed study. Upon further discussion of this point, all referees agreed with this suggestion. It is of course possible to add further data (e.g. along the lines proposed by referee 1). However, as we are all aware of competing manuscripts with similar findings, it might indeed be the most sensible solution at this point. Please let me know, if you do not agree and prefer to keep the interaction data in the current manuscript and we can discuss this further. Apart from this, please address the other remaining concerns from the referees.

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study.

- Please remove the dataset legends from the main text and add them either to a separate tab in the Data sets excel sheet or to the first line of the sheet.

- In case you decide to keep the proteomics data in the paper, please upload them to PRIDE (as indicated in the Author Checklist) and provide the accession code in a separate "Data Availability" section at the end of Materials & Methods (suggested wording: "The [protein interaction | microarray | mass spectrometry] data from this publication have been deposited to the [name of the database] database [URL] and assigned the identifier [accession | permalink | hashtag])."

- Statistics: I noticed that you calculated p-values in Fig. 2F, G, I, J; Fig. 3A, C, E, Fig. 4 and Fig. EV5C based on technical replicates. Please note that statistical tests should only be applied to biological replicates (see also Vaux et al, EMBO rep 2012). I therefore suggest removing the statistical data from these panels. I think that the effect size is convincing enough even in the absence of the proof of statistical significance.

- In Figure EV2 the blue magnification box is quite difficult to see. I suggest to either make the line thicker or to change the color.

- Figure legend for EV5A: you mention "data not shown". Please note that as per our editorial policy all data should be shown in the manuscript. Is it possible to include these control data?

- Thank you for supplying all Western blot source data: It appears however that the data for Fig. 5D are missing.

I look forward to seeing a final version of your manuscript as soon as possible.

****
REFEREE REPORTS

Referee #1:

In this revised version, Moretti and colleagues demonstrate the importance of TMEM41B, an ER-protein identified via a pool CRISP screening, in the autophagosome biogenesis/maturation sequence and show that TMEM41B KO had also consequences on lipid metabolism in the cell, notably for beta-oxidation. Overall the revised paper is well written: introduction presents nicely the open questions about autophagosome biogenesis sequence and membrane(s), and figures are informative. The part concerning the putative function of TMEM41B in autophagosome biogenesis has been considerably improved compared to the previous version of the manuscript: thus, the main message of the paper, concerning TMEM41B and autophagy, is better illustrated in this revised version. However, the paper general direction and conclusion(s) changed a lot: many previous data have been removed (including conclusions about VMP1 crosstalk with TMEM41B) and new data are shown to reinforce the hypothesis of TMEM41B possible role in lipid droplets dynamics. But the transition (and direct link) between autophagy part and LD part is weak in the text (not justified enough) and lacks appropriate and dedicated experiments. The notion of TMEM41B associated "mobilization" of lipids is often cited in the paper but is not very clear (is that direct? is that associated - or not - with functions in autophagosomal membrane biogenesis?). Thus, despite real improvement of the "autophagy related function of TMEM41B", the reported relationship of TMEM41B role in autophagosome biogenesis with contact sites membranes and/or lipid metabolism regulation is still speculative at this point.

MAJOR ISSUES:

Despite the CO-IP with SIGMAR1, the new data concerning interaction - and functional crosstalk - between TMEM41B and SIGMAR1 are a bit weak: are these proteins co-localized at subdomains of the ER, notably during autophagy induction? What is the effect of TMEM41B KO on SIGMAR1 function(s) and localization(s) and vice versa? Moreover, the control IP using empty MYC plasmid and anti-MYC immunoprecipitation must be shown to validate the specificity of the interaction.

MINOR COMMENTS:

- Results shown in Fig3A are puzzling: in the Bodipy-493 experiments, it seems to me that there is a difference between the control and the ATG7 KD conditions.

- Despite the illustration of LD defects in TMEM41B KO cells, the electron microscopy shown in Figure EV2 is not particularly helpful for autophagosome biogenesis defects phenotype: it is not really sufficient to demonstrate presence of "stalled autophagosome".

- It seems to me that the calnexin signal in Fig EV4B is saturated: is that on purpose?

- Justifying the use of H4 cells (for FACs based autophagy related read-outs for example), even in a short way, could be beneficial for readers.

Referee #2:

The authors have addressed all the points I raised in the initial review in a very satisfactory manner.

Referee #3:

This a re-review of the manuscript by Moretti et al., describing the role of TMEM41B in autophagy initiation as well as lipid transport between organelles. On the whole, my concerns have been adequately addressed and the manuscript is much more convincing. I feel the emphasis of the manuscript has changed slightly to focus in a little more detail on the lipid mobilization aspect and in particular membrane contact sites. I am fine with this (and still in favor of publication) but seeing as the authors now have a very nice way to detect endogenous TMEM14B (with the CRISPR myc KI), I think they should confirm that it really is at contact sites between the ER and forming autophagosomes/ER-mitochondria/ER-lipid droplets. I'm loath to suggest more experiments, but it should be straightforward to do some more immunofluorescence.

Other points:
Please include a higher magnification of the myc-KI TMEM41B images (Fig 5B), as it is hard to see the co-localization - a similar size image to Fig5A would help.

I feel the data on SIGMAR1 (Figure 5E and F) should be removed. I do not think it essential for this manuscript and it is too preliminary. While it correlates nicely with the lipid transport/contact site role of TMEM41B, more experimental work is required to confirm this - which is best left for a follow-up study.

Please include the MW markers on the immunoblots - the reader should not have to hunt through source data to find this important information - as well as being useful for looking at these proteins in their own experiments, it also quickly helps to validate that the authors are indeed looking at the right proteins themselves!

Referee #1:

In this revised version, Moretti and colleagues demonstrate the importance of TMEM41B, an ER-protein identified via a pool CRISP screening, in the autophagosome biogenesis/maturation sequence and show that TMEM41B KO had also consequences on lipid metabolism in the cell, notably for beta-oxidation. Overall the revised paper is well written: introduction presents nicely the open questions about autophagosome biogenesis sequence and membrane(s), and figures are informative. The part concerning the putative function of TMEM41B in autophagosome biogenesis has been considerably improved compared to the previous version of the manuscript: thus, the main message of the paper, concerning TMEM41B and autophagy, is better illustrated in this revised version. However, the paper general direction and conclusion(s) changed a lot: many previous data have been removed (including conclusions about VMP1 crosstalk with TMEM41B) and new data are shown to reinforce the hypothesis of TMEM41B possible role in lipid droplets dynamics. But the transition (and direct link) between autophagy part and LD part is weak in the text (not justified enough) and lacks appropriate and dedicated experiments. The notion of TMEM41B associated "mobilization" of lipids is often cited in the paper but is not very clear (is that direct? is that associated - or not - with functions in autophagosomal membrane biogenesis?). Thus, despite real improvement of the "autophagy related function of TMEM41B", the reported relationship of TMEM41B role in autophagosome biogenesis with contact sites membranes and/or lipid metabolism regulation is still speculative at this point.

MAJOR ISSUES:

Despite the CO-IP with SIGMAR1, the new data concerning interaction - and functional crosstalk - between TMEM41B and SIGMAR1 are a bit weak: are these proteins co-localized at subdomains of the ER, notably during autophagy induction? What is the effect of TMEM41B KO on SIGMAR1 function(s) and localization(s) and vice versa? Moreover, the control IP using empty MYC plasmid and anti-MYC immunoprecipitation must be shown to validate the specificity of the interaction.

As suggested by reviewer #3, we have decided to remove the SIGMAR1 data from the manuscript. We have kept the interaction proteomics data as supporting evidence for the ER localization of TMEM41B and as resource to guide future studies on the function of TMEM41B.

MINOR COMMENTS:

- Results shown in Fig3A are puzzling: in the Bodipy-493 experiments, it seems to me that there is a difference between the control and the ATG7 KD conditions.

We have quantified the total punctate area per cell over a large number of cells and there appears to be no statistical difference between NT and ATG7 KO conditions.

- Despite the illustration of LD defects in TMEM41B KO cells, the electron microscopy shown in Figure EV2 is not particularly helpful for autophagosome biogenesis defects phenotype: it is not really sufficient to demonstrate presence of "stalled autophagosome".

We agree that more in-depth studies, including immuno-EM, will be required for the precise characterization of these structures but this was not feasible in the timeframe of the revision. We present the data as supporting evidence for stalled autophagosomes and for the accumulation of lipid droplets in TMEM41B KO cells.

- It seems to me that the calnexin signal in Fig EV4B is saturated: is that on purpose?

Analysis of the pixel histogram of the calnexin channel shows homogenous distribution of pixel intensities, typical of properly exposed acquisitions.

- Justifying the use of H4 cells (for FACs based autophagy related read-outs for example), even in a short way, could be beneficial for readers.

The use of H4 cells is now justified at the beginning of the result section.

Referee #2:

The authors have addressed all the points I raised in the initial review in a very satisfactory manner.

We thank Referee #2 for the appraisal of our manuscript.

Referee #3:

This a re-review of the manuscript by Moretti et al., describing the role of TMEM41B in autophagy initiation as well as lipid transport between organelles. On the whole, my concerns have been adequately addressed and the manuscript is much more convincing. I feel the emphasis of the manuscript has changed slightly to focus in a little more detail on the lipid mobilization aspect and in particular membrane contact sites. I am fine with this (and still in favor of publication) but seeing as the authors now have a very nice way to detect endogenous TMEM14B (with the CRISPR myc KI), I think they should confirm that it really is at contact sites between the ER and forming autophagosomes/ER-mitochondria/ER-lipid droplets. I'm loath to suggest more experiments, but it should be straightforward to do some more immunofluorescence.

We thank Referee #3 for support of publishing this work. Localization of TMEM41B to contact sites will require a dedicated effort (high resolution microscopy, biochemical isolation of contact sites) which was not feasible in the timeframe of the revision.

Other points:

Please include a higher magnification of the myc-KI TMEM41B images (Fig 5B), as it is hard to see the co-localization - a similar size image to Fig5A would help.

Higher magnification micrographs for Figure 5B have been included.

I feel the data on SIGMAR1 (Figure 5E and F) should be removed. I do not think it essential for this manuscript and it is too preliminary. While it correlates nicely with the lipid transport/contact site role of TMEM41B, more experimental work is required to confirm this - which is best left for a follow-up study.

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Please include the MW markers on the immunoblots - the reader should not have to hunt through source data to find this important information - as well as being useful for looking at these proteins in their own experiments, it also quickly helps to validate that the authors are indeed looking at the right proteins themselves!

MW markers have been added to the immunoblot panels.

Accepted 12 July 2018

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REFEREE REPORT

Referee #1:

I think authors have been dealing with most of the referees' comments in adequate manner.

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Corresponding Author Name: Beat Nyfeler
Journal Submitted to: EMBO Reports
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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample sizes were chosen as large as possible while keeping experimental handling feasible.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were excluded from analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Subjective bias was minimized by analyzing cell lines in multi-well format using multichannel dispenser pipettes whenever feasible for data collection.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No blinding was applied. Subjective bias was minimized by applying automated microscopy and quantification whenever feasible for data analysis.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes, statistical tests are specified in the figure legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We assessed normality using Shapiro-Wilk test.
Is there an estimate of variation within each group of data?	No.
Is the variance similar between the groups that are being statistically compared?	The assumption of equal variance was not tested and therefore not included in the statistical analysis.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

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6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Catalog numbers of all antibodies are reported.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Done.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Interaction proteomics data uploaded to PRIDE and accession code listed in data availability section.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Mini-pool CRISPR screening data and interaction proteomics data are reported as datasets EV1 and EV2.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No.