Journal of Cell Science

TMEM147 interacts with lamin B receptor, regulates its localization and levels, and affects cholesterol homeostasis

Andri Christodoulou, Giannis Maimaris, Andri Makrigiorgi, Evelina Charidemou, Christian Lüechtenborg, Antonis Ververis, Renos Georgiou, Carsten W. Lederer, Christof Haffner, Britta Brügger and Niovi Santama
DOI: 10.1242/jcs.245357

Editor: Arnoud Sonnenberg

Review timeline
Original submission: 18 February 2020
Editorial decision: 13 March 2020
First revision received: 9 June 2020
Editorial decision: 29 June 2020
Second revision received: 1 July 2020
Accepted: 2 July 2020

Original submission

First decision letter

MS ID#: JOCES/2020/245357

MS TITLE: ER transmembrane protein TMEM147 interacts with Lamin B Receptor, regulates its levels and localization, and affects cholesterol biosynthesis

AUTHORS: Andri Christodoulou, Giannis Maimaris, Andri Makrigiorgi, Christian Luechtenborg, Antonis Ververis, Renos Georgiou, Carsten Werner Lederer, Christof Haffner, Britta Bruegger, and Niovi Santama
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. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. Important concerns are (1) the lack of specific controls in several experiments and (2) the consideration of an alternative explanation for the reduced expression of LBR upon knockdown of TMEM147. Thus, the reviewers find that additional experiments are required to support the role of TMEM147 in influencing LBR levels and LBR targeting to the INM. In major point 2 of reviewer 3, he/she probably refers to Fig. 5 and not Fig. 2.

If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers. Please ensure that you clearly highlight all changes made in the revised manuscript.

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

Lamin B receptor (LBR) is an extensively studied cellular protein that localizes to the inner nuclear membrane (INM). Two unrelated up to now functions have been attributed to LBR. Its N-terminal domain binds lamina and heterochromatin thus anchoring the nuclear envelope to an underlying scaffold, while its C-terminal transmembrane domains are involved in cholesterol biosynthesis. Mutations within the LBR molecule are associated with diseases such as the Pelger-Huët anomaly and Greenberg skeletal dysplasia. There are few reports on the regulation of LBR levels and even more interestingly on the possible co-ordination between its levels and its functions. In this manuscript, the authors present new findings showing that the ER transmembrane protein TMEM147 drastically influences LBR levels and LBR targeting to the INM, thus having an significant impact on cellular cholesterol levels.

Comments for the author

In this manuscript, Christodoulou et al. present evidence on the regulation of LBR and cholesterol biosynthesis by the ER transmembrane protein TMEM147.
The paper is well-written and interesting. However, I have some concerns that need to be addressed prior to publication.
Tsai et al. (ref. 21) did not observe any differences in the overall cell morphology or growth between WT and LBR KO cells under normal growth conditions. Only, when HeLa cells were cultured in lipoprotein-depleted medium the LBR KO cells exhibited slow growth, cell rounding, and detachment, followed by cell death. The sensitivity of LBR KO cells could be rescued by adding exogenous cholesterol to the medium. The authors observed decreased cell viability upon TMEM147-silencing at late stages post-transfection, even in the presence of complete medium. According to their hypothesis, this decreased cell viability may be due to changes in cholesterol metabolism. However, while they used lipid-restrictive medium (no serum) or the cholesterol transport inhibitor U-18666A to measure cholesterol uptake there is no mention of the viability of TMEM147-silenced cells under these growth conditions. Shouldn’t TMEM147-silenced cells be more sensitive in lipid-depleted medium? Could this decreased viability be reversed by the addition of exogenous cholesterol?
The low levels of LBR and DHCR7 in TMEM147-silenced cells are due to reduced transcription of the corresponding genes as clearly shown by RT-qPCR (Fig. 7C). TMEM147 silencing resulted in a robust reduction of endogenous LBR protein (Fig. 6A2, A3) and a reduction of LBR238-GFP that was not statistically significant (Fig. 6A2, A3). According to the authors this may be due to the reduced interaction of the N-terminal truncated LBR with TMEM147, suggesting that the interaction between the two proteins is the critical issue that regulates LBR stability. This suggestion contradicts the observation that TMEM147 downregulates LBR at the transcriptional level.
I think a more logical explanation for not observing a reduction of LBR238-GFP upon TMEM147-silencing is the lack of LBR promoter elements in the GFP plasmid.
Why only three bands were cut out of the gel shown in Suppl. Fig. 4? Based on what criteria were these bands selected? Were LBR and TM7SF2 among the immunoprecipitated bands? TM7SF2 displays significant sequence similarity with the C-terminal domain of LBR and it should also interact with TMEM147.
If such an interaction exists, this further strengthen the observation that TMEM147 knockdown downregulates LBR and DHCR7 at the transcriptional level while the gene expression of TM7SF2 remains unaffected.
LBR372-GFP and LBR209-615-GFP localize to the nuclear rim (appropriately is the word used by the authors) with some partitioning to the ER, similarly to full-length LBR-GFP (Fig. 6B2). According to the current state of knowledge and as the authors mention, LBR is anchored at the INM by binding,

via its N-terminus, to the nuclear lamina and heterochromatin. Shouldn't LBR209-615-GFP exhibit an increased ER partitioning?
The quality of the blot shown in Fig. 6B5 should be improved.

Reviewer 2

Advance summary and potential significance to field

Christodoulou and colleagues report on the interaction of the ER transmembrane protein TMEM147 with lamin B receptor (LBR) which in addition to its duties in the inner nuclear membrane also serves as a sterol reductase in cholesterol synthesis. The authors show that tagged TMEM147 is indeed localized in the ER (Fig 1), and that knocking it down with siRNA has a striking effect on reducing LBR protein levels (Fig 3). What little LBR is left after TMEM147 knockdown is preferentially localized to the ER (Fig 4) rather than its usual residence of the inner nuclear membrane. Moreover, IP experiments indicate that ectopic TMEM147 binds to endogenous or tagged LBR (Fig 5). Specifically, TMEM147 interacts with the C-terminal portion of LBR (Fig 6). TMEM147 knockdown also reduces another sterol reductase DHCR7 (Fig 7), and apparently this by downregulating the genes for both LBR and DHCR7 (but not that of another sterol reductase TM7SF2). Furthermore, TMEM147 knockdown increases cell free cholesterol and cholesterol uptake but reduces cholesteryl esters, which is somehow used to infer reduced cholesterol synthesis.

Comments for the author

The link between TMEM147 and LBR is very interesting, but both the mechanism and functional consequences require more work.

Major comments
1. How does knocking down TMEM147 downregulate the genes for both LBR and DHCR7 but not that of another sterol reductase TM7SF2? This mechanism is at present completely unexplored and unexpected, especially considering that DHCR7 and TM7SF2 are similarly transcriptionally regulated whereas LBR is not (Please refer to a recent paper which discusses this - Capell-Hattam et al. JBC 2020). So is the effect of TMEM147 purely transcriptional and not post-translational? e.g. Does knocking down TMEM147 affect FLAG-LBR levels (used in Fig 5)?

2. How does the increase in cell free cholesterol and cholesterol uptake but reduced cholesteryl esters infer reduced cholesterol synthesis? Firstly, on page 13, it is stated that "CE levels give a measure of cellular cholesterol biosynthesis…" How? What is the evidence for this? Secondly, the cellular uptake of fluorescently tagged-cholesterol does not address whether or not TMEM147 affects cholesterol synthesis. Moreover, if measuring cholesterol uptake was the primary goal, you would need to assess lipoprotein-associated cholesterol uptake (presumably via LDLR). I don't think this assay is particularly useful here. Since the authors wish to make inferences about affects on cholesterol synthesis, this is the parameter they should be directly measuring.

Specific comments
1. In Fig 5A, what are the bands in the TMEM147-GFP lanes that are bigger than GFP alone?
2. In Fig 5B, why is does the FLAG-LBR run higher in the bound Co-IP lane?
3. Fig 5: can you show this interaction for endogenous TMEM147?
4. Fig 6A1,B1: It is hard to see the numbers for the TMs? I thought ref#16 proposed 10TMs based on homology modelling?
5. Supp Fig 5: For the unsaturated CEs, please give the double bond position e.g. n-9, n-6, n-3.

Reviewer 3

Advance summary and potential significance to field

If further evidence in support of the authors conclusions can be obtained, the study at hand will establish a new role for TMEM147 in the context of LaminB receptor localization and cholesterol metabolism, which would be of interest to the readership of JCS.

Comments for the author

Christodoulou et al. report on the characterization of the transmembrane protein TMEM147 and suggest a functional role in the context of LaminB receptor stability and localization as well as functional ties to cholesterol metabolism.
Overall, many of the presented data that support the conclusions have a somewhat preliminary character. To make a convincing case, the authors should consistently include adequate specificity controls and attempt to identify cellular phenotypes prior to the manifestation of overt viability defects. Furthermore, too many alternative interpretations are still possible and need to be explored or ruled out by experiment. Specific suggestions, appended below, might be useful to solidify TMEM147's suggested role in LBR trafficking/localization and cholesterol biosynthesis.

Major points

1) According to Fig. 3A, cell viability is reduced by ~50% 72 h after TMEM147 silencing. Can the authors exclude the possibility that the observed morphological/functional changes in several subsequent figures/experiments are merely due to cellular toxicity imposed by the knockdown? Are these effects visible at 48h post transfection with siRNA prior to the onset of overt viability issues? This would potentially be an easy way to resolve this concern.

2) Fig. 2 –It would be useful to include an irrelevant transmembrane or polytopic protein as specificity control. This would exclude the formal possibility that the "interaction" is attributable to two transmembrane proteins residing in a shared detergent micelle (a common artifact in co-IPs w/ detergent-solubilized membrane proteins).

3) Fig. 7A. Again, a specificity control would be useful. Are only the proteins of interest affected or does TMEM147 silencing lead to a general folding defect of polytopic membrane proteins?

4) Fig. 4 C: Is it indeed the LBR distribution that changed or are the observed differences attributable to a reduced signal/noise ratio due to lower LBR levels? One easy way to check this would be to knock down LBR, and generate a 3D rendering for cells with reduced LBR levels. Furthermore, the authors state that they observe chromatin decondensation (bottom of p. 10) but don't call out a figure. What are the data supporting this observation?

5) If the cells are compromised in synthesizing cholesterol, the SREBP pathway should be turned on, and TM7SF2 should be strongly upregulated (Bennati et al., PMID: 16784888; Tsai et al., PMID: 27336722), even if futile due to absence of LBR). However, TM7SF2 is not upregulated (cf. Fig. 7C). Can the authors explain why not? This is a rather puzzling finding. OR does an induction of cholesterol uptake upon TMEM147 silencing "preemptively" obviate the need for cholesterol synthesis? The authors should address this point by comparing transcript levels (+/-TMEM147 knockdown) both in normal medium and under cholesterol starvation conditions where uptake cannot compensate for a possible synthesis deficiency.

6) the authors should address the possibility that TMEM147 knockdown leads to the induction of the unfolded protein response, causing translational downregulation of membrane proteins (LBR etc) resulting in destabilization of messages specifying ER proteins and the induction of lipid synthesis for ER expansion. Is the change in lipid composition specific to cholesterol/cholesterol esters or are phospholipids levels also higher? A standard lipid profile would be useful.

Minor points

1. Page 9, the authors *state "TMEM147-GFP displayed the same distribution as the Flag-tagged version"*. They actually do not look the same. Comparing Fig. 1 A1 and B1, the GFP-tagged TMEM147 does not effectively localize to the nuclear envelope, and does not co-localize with Lap2beta (INM marker) in Fig.1 D3. Does the GFP tag affect the distribution of TMEM147?

2. Fig. 6, Panel B5. The quality of the TMEM147 blot is suboptimal, this experiment should be repeated and a specificity control included (e.g. using an antibody against an irrelevant polytopic protein).

3. The authors state that TMEM147 and DHCR7 interact. How exactly was the IP/MS experiment performed? The IP looks far from clean (not unusual for detergent extracts/membrane protein IPs), normally 100s of proteins would be detected even in an excised gel band with any contemporary standard MS setup. Therefore, the sequence coverage of DHCR7 should be shown, along with a rank list of identified proteins. The authors should confirm this IP via IP/Western blotting and importantly, using suitable controls (GFP is not a suitable control, cf. major point 2). Alternatively, all DHCR7 data could simply be omitted.

a) In Fig, 7E (and corresponding text sections) is it really absorbance that is monitored or rather fluorescence?

b) In the discussion (2nd paragraph), the author state that changes in cholesterol metabolism imposed by TMEM147 depletion are responsible for the observed toxicity. This interpretation seems unlikely since HeLa LBR KO cells grow normally in regular medium unless they are shifted to cholesterol-restrictive conditions. Even then, the growth defects can be rescued by addition of LDL or cholesterol (cf. Tsai et al., PMID: 27336722). It seems equally likely that other defects account for the observed toxicity. This could be discussed or addressed experimentally.

c) Page 13, *"At the same time, total levels of cholesteryl esters (CE) were greatly reduced, by 68% (5.80±1.68 mol% in TMEM147-silenced cells vs. 9.74±1.83 in control"*. 9.74% to 5.8% is not equal to "reduced by 68%".

First revision

Author response to reviewers' comments

Point-by Point Response

We would like to thank all the Reviewers for their constructive criticism (blue font), which we took to heart to address. Below are our detailed responses.

Comments by Reviewer 1

In this manuscript, Christodoulou et al. present evidence on the regulation of LBR and cholesterol biosynthesis by the ER transmembrane protein TMEM147. The paper is well- written and interesting.

We thank the Reviewer for the positive appraisal.

1) However, I have some concerns that need to be addressed prior to publication. Tsai et al. (ref. 21) did not observe any differences in the overall cell morphology or growth between WT and LBR KO cells under normal growth conditions. Only, when HeLa cells were cultured in lipoprotein-depleted medium the LBR KO cells exhibited slow growth, cell rounding, and detachment, followed by cell death. The sensitivity of LBR KO cells could be rescued by adding exogenous cholesterol to the medium. The authors observed decreased cell viability upon TMEM147-silencing at late stages post- transfection, even in the presence of complete medium. According to their hypothesis, this decreased cell viability may be due to changes in cholesterol metabolism. However, while they used lipid-restrictive medium (no serum) or the cholesterol transport inhibitor U-18666A to measure cholesterol uptake there is no mention of the viability of TMEM147-silenced cells under these growth conditions. Shouldn't TMEM147- silenced cells be more sensitive in lipid-depleted medium? Could this decreased viability be reversed by the addition of exogenous cholesterol?

(a) Indeed, as the Reviewer points out, Tsai *et al.*, (2016) observed that decreased growth and viability of LBR KO cells occurred under lipid restriction. In our case, decreased viability is manifested already in complete medium in cells that are silenced for *TMEM147* (which results in concomitant *LBR* silencing). We believe that the phenotypes we obtain are more severe with *TMEM147* silencing

because, as shown in our manuscript in Fig. 7A-C (and now even more clearly with the new experiment provided in new Suppl. Fig. S5 in response to the request of Reviewer 3, major point 5), *TMEM147* silencing also downregulates expression of additional sterol reductase *DHCR7*. Therefore, cells suffer from the combined effect of loss of both critical enzymes. As shown in Fig. 7D, upon *TMEM147* silencing cholesterol uptake is elevated both in complete and lipid restricted media, implying increased need for cholesterol even under normal conditions.

(b) Inspired by the Reviewer's comment and to directly address his/her important questions, we have now designed and **conducted a new set of experiments** in which cell viability and the effect of the addition of exogenous cholesterol were assessed under different conditions in parallel (**shown in revised Fig. 7 new panels F, G, and Results section p.16**). Control and *TMEM147*-silenced cells were grown either in full medium or in full medium followed by a 24-hour lipid restriction (no serum) either on its own or in combination with exogenous cholesterol and cell viability was monitored at different time points. Addition of exogenous cholesterol for the last 8hr during the 24-hour lipid restriction rescued growth and restored it by 72hr to the same levels as those observed at 48hr specifically in *TMEM147*-silenced cells. In comparison, equivalent *TMEM147*-silenced cells at 72 hr without cholesterol showed the typical drastic reduction in viability. Cholesterol addition had only a very modest positive effect on lipid-restricted control cells.

2) The low levels of LBR and DHCR7 in TMEM147-silenced cells are due to reduced transcription of the corresponding genes as clearly shown by RT-qPCR (Fig. 7C). TMEM147 silencing resulted in a robust reduction of endogenous LBR protein (Fig. 6A2, A3) and a reduction of LBR238-GFP that was not statistically significant (Fig. 6A2, A3). According to the authors this may be due to the reduced interaction of the N-terminal truncated LBR with TMEM147, suggesting that the interaction between the two proteins is the critical issue that regulates LBR stability. This suggestion contradicts the observation that TMEM147 downregulates LBR at the transcriptional level. I think a more logical explanation for not observing a reduction of LBR238- GFP upon TMEM147-silencing is the lack of LBR promoter elements in the GFP plasmid.

(a) Given the effect of *TMEM147* silencing on the transcription of both *LBR* and *DHCR7*, the Reviewer's comment is indeed a very logical explanation and **we have now added** this point to the pertinent Results section (p.13-14). The inserted sentence reads *"We note however the lack of LBR promoter elements driving the expression of LBR238-GFP in the stable cell line, which would explain the lack of effect if regulation was only at the transcriptional level."*

(b) Given that TMEM147 also has a physical interaction with LBR (as documented in Fig. 5 and revised Fig. 6 B5 lane i), it is extremely likely that protein-protein interactions play critical roles in protein stability (and activity) of LBR in addition to its transcriptional regulation. This idea also seems to be consistent with the effect of TMEM147 on protein stability in other protein complexes it forms, specifically with proteins Nomo and Nicalin. As one of us previously showed (Dettmer *et al.*, 2010), the knockdown of TMEM147, Nicalin or NOMO, resulted in a strong reduction of the protein levels of the other binding partners without affecting their mRNA levels. All the above, make our experiments in Fig. 6, and especially the one in panel B5 (now replaced with a good image), all the more necessary in our investigation. In conclusion, we believe that effects on transcription and protein stability are not mutually exclusive and a combination of transcriptional regulation and protein-protein interactions is important in the functional interaction between TMEM147 and LBR and the resulting phenotypes upon silencing of *TMEM147*.

3) Why only three bands were cut out of the gel shown in Suppl. Fig. 4? Based on what criteria were these bands selected? Were LBR and TM7SF2 among the immunoprecipitated bands? TM7SF2 displays significant sequence similarity with the C-terminal domain of LBR and it should also interact with TMEM147. If such an interaction exists, this further strengthen the observation that TMEM147 knockdown downregulates LBR and DHCR7 at the transcriptional level, while the gene expression of TM7SF2 remains unaffected.

We apologise for the manner the IP/MS experiment was presented before: we had chosen the 3 bands in comparison to a parallel IP with unrelated ER protein TMEM170 but this was not shown/mentioned in the Figure and was clearly not effective.

To address the important comment of Reviewer 1, but also a similar comment made by Reviewer 3

(minor point 2), **we have now conducted a brand-new IP/LC-MS analysis, which we show in the new Suppl. Fig. S4.**
The analysis included, in addition to the TMEM147-GFP test IP, a GFP-only negative-control IP, and an IP with ER transmembrane protein TMEM129-GFP for comparison/validation. The samples from 2 independent sets of experiments (6 IPs in total) were processed, differentially labelled, and extracted peptides were pooled and analysed in a common LC/MS run. The results (summarized in the new Suppl. Fig. S4) showed:

(a) That all three reductases, namely LBR, DHCR7 and TM7SF2 were identified as enriched hits for interaction with TMEM147. This confirmed the Reviewer expectation about TM7SF2. In fact, the conclusion was further strengthened by another new experiment we conducted in order to measure the mRNA levels for all Reviewer 3, major point 5 (new Suppl. Fig. 5);

(b) Gene Ontology analysis of hits resulted in the identification of pertinent GO terms for biological processes that were appropriate for each of the TMEM proteins. For TMEM147, "*lipid metabolism*", "*membrane lipid biosynthesis*" and "*sterol metabolism*" were among the top GO terms for biological process, consistent with our findings. For TMEM129 (an ERAD E3 ubiquitin ligase, essential for virus-induced degradation of MCH-I), top GO terms included "*SCF-dependent proteasomal ubiquitin-dependent protein catabolic process*" and "*antigen processing and presentation of peptide antigen via MHC class I*", again consistent with its published functions and thus validating our results.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (EMBL-EBI) with the dataset identifier PXD019598.

4) LBR372-GFP and LBR209-615-GFP localize to the nuclear rim (appropriately is the word used by the authors) with some partitioning to the ER, similarly to full-length LBR-GFP (Fig. 6B2). According to the current state of knowledge and as the authors mention, LBR is anchored at the INM by binding, via its N-terminus, to the nuclear lamina and heterochromatin. Shouldn't LBR209-615- GFP exhibit an increased ER partitioning? The quality of the blot shown in Fig. 6B5 should be improved.

(a) The LBR209-615GFP construct includes the 1st TM domain and can thus be targeted to the NE (shown in **Fig. 6B3**).

(b) We apologise for the bad quality of the TMEM147 strip in the Western blot of Fig. 6B5. **We have repeated the experiment** and now provide a good image for the whole set, in **revised Fig. 6B5**.
Over and above this request, we also provide **additional supportive data** in **new panel C** of revised Suppl. Fig. S3, showing that the other N-terminal construct, HeLa-LBR238-GFP, was also unable to bind the native TMEM147, as was the GFP-only bait, used as a negative control (Results, p.14).

Comments by Reviewer 2

Major comments
1) How does knocking down TMEM147 downregulate the genes for both LBR and DHCR7 but not that of another sterol reductase TM7SF2? This mechanism is at present completely unexplored and unexpected, especially considering that DHCR7 and TM7SF2 are similarly transcriptionally regulated whereas LBR is not (Please refer to a recent paper which discusses this - Capell-Hattam et al. JBC 2020).
So is the effect of TMEM147 purely transcriptional and not post-translational? e.g. Does knocking down TMEM147 affect FLAG-LBR levels (used in Fig 5)?

As the Reviewer rightly mentions, gene expression of *LBR* and *TM7SF2* is not co-ordinately regulated,
(a) as documented in literature (for example Bennati *et al.*, 2008; Tsai *et al.*, 2016; Bartoli *et al.*, 2016) and summarized in our Discussion (p.17-18),
(b) as shown in the suggested Capell-Hattam *et al.*, 2020 publication, and
(c) as shown by our own results (Fig. 7C) indicating co-ordinate transcriptional response of *TMEM147* and *LBR*, but not of *TM7SF2*.
Thus, the differential regulation of *LBR* and *TM7SF2* has been established in many studies so far, including in the Capell-Hattam study (in which TM7SF2 is mentioned with the alternative name DHCR14). In response to the Reviewer comment, we have now also **added reference** to the Capell-

Hattam *et al.* study of January 2020 (which had not come to our attention at the time of submission) in our Discussion (p.16-17 red passage).

The mechanism of regulation of sterol reductases in cholesterol biosynthesis appears to be both transcriptional and post-translational (for example effects via ubiquitination/the proteasome as per Capell-Hattam *et al.*). In our work, we showed that *TMEM147* downregulation also affects transcription levels of *DHCR7*. In studies from the A.J Brown group (Prabhu *et al.*, 2014), *DHCR7* and *TM7SM2* show co-ordinate transcriptional regulation (which was not what we observed in our experiments), and DHCR7 is also liable to post-translational regulation (Prabhu *et al.*, 2016). However, in these studies measurements were made with stable (unaltered) concentration of LBR, which is not the case in our experiments, in which TMEM147 reduction results in drastic reduction of LBR levels as well. Thus, the difference in results regarding DHCR7 and TM7SF2, pointed out by the Reviewer, does not apply to readily comparable situations. This may indicate that LBR is functionally epistatic and/or that TMEM147 is at the top of the functional hierarchy, modulating cholesterol synthesis*. Because we have not directly addressed protein stability of LBR (by looking at FLAG-LBR levels during TMEM147 silencing or in other ways) or of DHCR7, we did not comment or speculate on this aspect. The demonstration of pairwise physical interactions between TMEM147 and LBR or DHCR7 proteins and previous literature on TMEM147, showing its effects on protein stability, but not transcription levels, of other interacting partners (Dettmer *et al.*, 2010; Rosemond *et al*, 2011), make additional post-translational effects likely.

* A **new experiment** (**new Suppl. Fig. S5**) that we conducted in response to the request of Reviewer 3, major point 5, **directly addresses** this specific issue raised here and further substantiates this idea, so that we would like to also refer you to our response to this point (p.9- 10 of this letter).

2) How does the increase in cell free cholesterol and cholesterol uptake but reduced cholesteryl esters infer reduced cholesterol synthesis? Firstly, on page 13, it is stated that "CE levels give a measure of cellular cholesterol biosynthesis…" How? What is the evidence for this? Secondly, the cellular uptake of fluorescently tagged-cholesterol does not address whether or not TMEM147 affects cholesterol synthesis. Moreover, if measuring cholesterol uptake was the primary goal, you would need to assess lipoprotein-associated cholesterol uptake (presumably via LDLR). I don't think this assay is particularly useful here. Since the authors wish to make inferences about affects on cholesterol synthesis, this is the parameter they should be directly measuring.

(a) Our comment on reduced cholesteryl esters was made to simply refer to the fact that esters are a storage form of cholesterol and that their levels (high or low) reflect the availability of intracellular cholesterol. Thus, reduction of esters (Fig. 7D) implies paucity of free cholesterol, and it is not unreasonable to think that increased cholesterol uptake (Fig. 7E) may be a counteractive mechanism to balance it. **We slightly reworded the sentence** (p.15, first sentence of last paragraph) for clarity.

(b) Regarding the uptake assay, we would like to highlight that fluorescently labelled cholesterol (NBD-cholesterol) is in fact taken up by cells via receptor-mediated vesicular uptake, using the high-density lipoprotein (HDL) receptor SRB1 (Florov *et al.*, 2000). While the LDR-receptor mediated endocytosis is the best-characterized uptake mechanism in hepatocytes and few other cell types, HDL-uptake is probably the most commonly utilized system by almost all cell types, including HeLa cells, which highly express SRB1 (The Human Protein Atlas https://www.proteinatlas.org/ENSG00000073060-SCARB1/cell). SRB1's apoprotein specificity is wide because, although it primarily binds HDL, it also binds LDL and VLDL-cholesterol as well. Furthermore, NBD-cholesterol does not only employ the HDL- receptor uptake system but, once internalized, it is rapidly esterified in the same pathway as native free cholesterol (Florov *et al.*, 2000). Because of these features that mimic cholesterol, NBD-cholesterol is an established probe for examining lipoprotein-mediated cholesterol uptake *in vivo* and in cultured cells (Huang *et al.*, 2015 and references within). **We added a brief reference** to this information in Materials and Methods (p.10). In conclusion therefore, we remain confident in the uptake assays in Fig. 7E, the results of which are clear cut and compelling.

Specific comments

1. In Fig 5A, what are the bands in the TMEM147-GFP lanes that are bigger than GFP alone?

These bands (seen in both the input and bound lanes) correspond to an internal initiation product that is an occasional occurrence with expression driven by the pEGFPN1 plasmid vector. Specifically, there is a methionine residue in the TMEM147 sequence, 21 aa upstream its C-terminus; together with the plasmid backbone up to the in-frame start of the GFP, would result in a product about 4 kDa larger than GFP alone. This would be very compatible with the extra band we observe in the WB.
For clarity to the reader, **we have now indicated the presence of this band** in revised Fig. 5 and added the pertinent information (internal initiation product) to the figure legend.

2. In Fig 5B, why is does the FLAG-LBR run higher in the bound Co-IP lane?

In our experience and generally speaking, bound fractions do have the tendency to run slightly higher, possibly due to high-salt extraction from the beads that affects their electrophoretic mobility.

3. Fig 5: can you show this interaction for endogenous TMEM147?

In Fig. 5 we show interaction between TMEM147-GFP+native LBR (panel A) and TMEM147- GFP+FLAG-LBR (panel B), whereas the interaction with **native TMEM147**, as the Reviewer is requesting, **is instead shown in Fig. 6B5 (lane i).** In the revised version of Fig. 6, panel B5 has been replaced with a much better image than before and the result is clear so that we hope to have covered this point.

4. Fig 6A1,B1: It is hard to see the numbers for the TMs? I thought ref#16 proposed 10TMs based on homology modelling?

The structural modeling and membrane topology of LBR is not a settled issue (as mentioned in the *Introduction* p.3). There are models:
(a) mostly proposing 8 or 9 TM domains in LBR (for example, Ye and Worman 1994, Olins *et al*. 2010, Zwerger *et al*. 2010), or, as the Reviewer mentions,
(b) more recently even 10 TM domains, as the Reviewer reminds us, based on the solved structure of Δ(14)-sterol reductase (MaSR1) from the bacterium *M. alcaliphilum* (Li *et al.*, 2015 and also shown as Fig. 4A in Tsai *et al.*, 2016).

For the purpose of illustrating LBR in Fig. 6, we relied on the sequence analysis currently offered by Uniprot for LBR with 8TMs (https://www.uniprot.org/uniprot/Q14739), after having made the qualifying statement in *Introduction*.

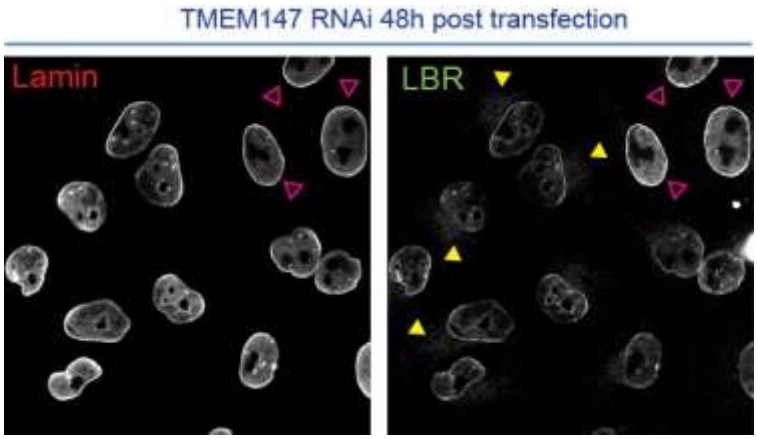
5. Supp Fig 5: For the unsaturated CEs, please give the double bond position e.g. n-9, n-6, n- 3.
Unfortunately, the method used does not determine the double bond positions in unsaturated CEs and therefore we cannot provide this information (now Suppl. Fig. 6).

Comments by Reviewer 3

Major points

1) According to Fig. 3A, cell viability is reduced by ~50% 72 h after TMEM147 silencing. Can the authors exclude the possibility that the observed morphological/functional changes in several subsequent figures/experiments are merely due to cellular toxicity imposed by the knockdown? Are these effects visible at 48h post transfection with siRNA prior to the onset of overt viability issues? This would potentially be an easy way to resolve this concern.

While we cannot readily exclude cellular toxicity, we do in fact see the defining results of silencing (notably LBR reduction), already developing at earlier time points (for example at 48h post transfection). We show below, for the Reviewers' inspection, confocal images, similar to those displayed in our Fig. 3 (panels D1-D6), but taken at 48h post transfection. As can be observed, the LBR signal is reduced while Lamin A is unaffected in silenced cells.



Reduction of LBR labeling is clearly detectable already at 48h post-transfection (observe, for contrast, a cluster of non-silenced cells indicated with magenta arrowheads). Upon inspection, LBR mistargeting to the ER is also visible in silenced cells (yellow arrowheads).

To clarify this point for the reader, **we have now added this phrase** to the manuscript:

"In addition, the most striking result upon TMEM147 silencing was our observation of a concomitant drastic reduction of levels of the INM protein lamin B receptor (LBR), already detectable at earlier time points" (p.11).

2) Fig. 2 –It would be useful to include an irrelevant transmembrane or polytopic protein as specificity control. This would exclude the formal possibility that the "interaction" is attributable to two transmembrane proteins residing in a shared detergent micelle (a common artifact in co-IPs w/ detergent-solubilized membrane proteins).

Maybe the Reviewer refers to Fig. 5 here? If so, please see **our response and revision as described under minor point 3** (and also point 3 of Reviewer 1)

3) Fig. 7A. Again, a specificity control would be useful. Are only the proteins of interest affected or does TMEM147 silencing lead to a general folding defect of polytopic membrane proteins?

We are confident that the effects we observe upon *TMEM147* silencing with reduction of both *LBR* (Fig. 3) and *DHCR7* (Fig. 7) are specific and not an effect of ER stress, leading to the unfolded protein response (UPR), alluded by the Reviewer here and also mentioned specifically in major point 6.
In particular,
(a) when we probed the same samples as shown in Fig. 3 with an antibody to the folding chaperon calnexin, an integral ER protein that is induced by ER stress and contributes to apoptotic cell death (thus serving as an ER stress protein marker, Guerin *et al.*, 2008), there was no notable change in calnexin levels in silenced *vs.* control cells. This would indicate no ER stress and absence of the associated UPR (causing enhanced proteolysis and reduction in protein translation), resulting from TMEM147 depletion.
(b) Furthermore, probing the same samples with an antibody to emerin, another NE transmembrane protein (like LBR), revealed no changes in its protein levels (contrary to LBR), strengthening our conclusion for a specific effect of silencing on LBR.

In this context we should also point out **revised Fig. 3**, although not specifically mentioned in the Reviewer's comment, because we believe that data shown there address the substance of the comments here and, in part, in point 6. In **revised Fig. 3,** we now include quantification of additional ER proteins **(calnexin; emerin) by western blot for the same 3 independent experiments, as described in its associated legend (p.28) and Results text (p.11).**

Regarding Fig. 7, as the Reviewer requests, **we have now also added this specificity control** to show that TMEM147 silencing does not lead to a general folding defect and reduction of several/unrelated ER proteins. In **revised Fig.7A, an additional WB experiment in the same samples**, displays no significant change in the ER transmembrane protein LEM4. Appropriate changes were also made in Fig. 7 legend (p.31).

4) Fig. 4 C: Is it indeed the LBR distribution that changed or are the observed differences attributable to a reduced signal/noise ratio due to lower LBR levels? One easy way to check this would be to knock down LBR, and generate a 3D rendering for cells with reduced LBR levels.

We believe that, with the data in hand, it is extremely unlikely that the ER-associated labeling we observe after TMEM147 silencing is chronic noise in LBR distribution that only becomes apparent because of diminished LBR signal at its main NE localization.

(1) All image acquisition for the total of 103 cells in 4 independent experiments, was obtained using identical settings (laser intensity and duration) for the subsets of control and silenced samples and are therefore directly comparable. Any ER localization (whether corresponding to fractional signal or to noise) would therefore be equally visible in control and silenced samples, and the numbers in Table S1 (pasted below for convenience) do not show this to be the case. What the numbers of our analysis shows is that ER localization is detectable in negative control cells (but in only 6 of 55 cells), while in TMEM147-silenced cells this occurs in 31 of 48 cells. We did not want to elaborate that point in the manuscript, but the odds ratio for this distribution is extremely significant: **Fisher's exact test, P value <0.0001 (GraphPad Prism).**

Data analyzed	Control - Silencing	TMEM147_siRNA	Total cells
ER+	**6**	**31**	37
ER-	**47**	**15**	62
Unclear	2	2	4
Total	55	48	103

There is therefore an extremely significant difference in frequency of ER detection under identical acquisition settings within the linear range of the imaging equipment.

(2) It is known and documented that, although the vast majority of LBR accumulates at the NE, a small fraction can be found at the ER (Holmer *et al.*, 1998; Clayton *et al.*, 2010; Nikolakaki *et al.*, 2017). Such presence of LBR at the ER may merely reflect its transient detection at the site of synthesis before localization, it may reflect a minority specific localization and biological role of LBR at ER, or it may reflect a random steady-state distribution of a fraction of LBR molecules. This raises two points. First, those third-party data show that LBR localization to the ER is a widely acknowledged phenomenon. Second, in the third case of random steady-state distribution and because of the nature of random distributions such as diffusion, LBR would be even less detectable at the ER for lowered LBR levels, making the observation of increased ER detection for our experiments even more remarkable.

In conclusion, we believe that the shift of LBR to the ER is a real phenotype with biological significance that is associated with TMEM147 silencing. Given the other data presented in the paper, including interaction data, the most probable mechanistic explanation of this change in distribution is the lack of interactions with TMEM147, via the C-terminus of LBR, that contribute to LBR's diffusional motility and NE targeting.

Furthermore, the authors state that they observe chromatin decondensation (bottom of p. 10) but don't call out a figure. What are the data supporting this observation?

In Fig. 4 panels A3+A4 and quantification in panel B2 and associated Results text (now p.12), we had shown the reduction in Hoechst incorporation, as a marker of chromatin compaction. These are the data referred to in Discussion (p.19).
Nevertheless, prompted by the Reviewer's comment and to further strengthen this point, **we now have conducted a new experiment** where we used the modification of histone H3, trimethylated H3K9, as an additional marker for heterochromatin. As we know, LBR is important in heterochromatin organization at the nuclear lamina, including interactions with histones H3 and H4 (Solovei *et al.*, 2013). A general hallmark of transcriptionally silent heterochromatin is methylation of histone H3 on lysine 9 (H3K9), which is also a marker for chromatin anchoring to the lamina (reviewed by Mattout *et al.*, 2015). In our immunofluorescence experiment with anti-H3K9me3, we observe a clear and

TMEM147- silencing-specific reduction of H3K9me3-labelled chromatin masses, relative to negative control cells. **This corroborating result is integrated in revised Fig. 4 as panels A5+A6 with corresponding changes in the legend and mention in the Results section** (p.12) and is consistent with our statement about chromatin decondensation upon TMEM147 silencing (and concomitant LBR downregulation).

5) If the cells are compromised in synthesizing cholesterol, the SREBP pathway should be turned on, and TM7SF2 should be strongly upregulated (Bennati et al., PMID: 16784888; Tsai et al., PMID: 27336722), even if futile due to absence of LBR). However, TM7SF2 is not upregulated (cf. Fig. 7C). Can the authors explain why not? This is a rather puzzling finding. OR does an induction of cholesterol uptake upon TMEM147 silencing "preemptively" obviate the need for cholesterol synthesis? The authors should address this point by comparing transcript levels (+/- TMEM147 knockdown) both in normal medium and under cholesterol starvation conditions where uptake cannot compensate for a possible synthesis deficiency.

Our findings that *TM7SF2* is not upregulated upon *TMEM147* silencing and concomitant *LBR* reduction is somewhat counterintuitive (but in fact entirely consistent with all the reports for non-co-ordinated transcriptional regulation between *TM7SF2* and *LBR*-see our answer to major point 1 of Reviewer 2). It may indeed be explained by the availability of external cholesterol and increased uptake as a compensatory mechanism under normal conditions.

Taking up the Reviewer's helpful advice, **we have now conducted the requested new experiment (presented in new Fig. S5)** where we quantify *TM7SF2* gene expression in either +/- *TMEM147* and both in normal medium and under cholesterol starvation conditions. **We expanded** the experiment suggested by the Reviewer to **also look** at what happens to *LBR* and *DHCR7* at the same time. Our findings indicate that:

(a) As expected (Bennati *et al.* 2006; Tsai *et al.* 2016), we found that negative control cells upregulated *TM7SF2* under serum starvation (lipid restriction) and also found that *TMEM147*- silenced (and therefore *LBR* downregulated) cells equally induced *TM7SF2*. Specifically, we did not observe a significant quantitative difference between negative control cells and *TMEM147*-silenced (*LBR* downregulated) cells in their ability to upregulate *TM7SF2* under lipid restriction (**new** Fig. S5C), exactly as observed by Tsai *et al.* (their Fig. 3 suppl.1).

(b) Again, in complete agreement with Tsai *et al.* (their Fig. 3 suppl.1), we found *LBR* to be constitutively expressed and unresponsive to lipid starvation (Fig. S5B). And we had exactly the same result with the expression of *TMEM147* (Fig. S5A).

We also expanded our analysis by including *DHCR7* in this new experiment. Interestingly, we found that while negative control cells also appear to strongly induce *DHCR7* expression upon lipid restriction, *TMEM*-silencing (*LBR* downregulation) appears to reduce *DHCR7* levels significantly, replicating the trend of reduction seen in non-restrictive conditions in silenced cells. The reduced *DHCR7* levels in *TMEM147*-silenced cells are, however, comparable to negative control levels in normal, non-restrictive conditions (Fig. S5D) and we can only speculate that this, combined with increased uptake (Fig. 7E), may help alleviate somewhat cholesterol paucity resulting from *TMEM147* silencing. Especially because, as Tsai *et al.* have shown, LBR is essential for cholesterol synthesis despite the presence of TM7SF2.

We have appropriately **revised** the Results section (p.15; **new Fig. S5**) and have made an **addition** to the Discussion (p.18) to include the information from this additional experiment.

6) the authors should address the possibility that TMEM147 knockdown leads to the induction of the unfolded protein response, causing translational downregulation of membrane proteins (LBR etc) resulting in destabilization of messages specifying ER proteins and the induction of lipid synthesis for ER expansion. Is the change in lipid composition specific to cholesterol/cholesterol esters or are phospholipids levels also higher? A standard lipid profile would be useful.

We have now experimentally addressed the possibility of the unfolded protein response (UPR) by including, with tools that were available to us, specificity controls in Fig. 3 (ER protein calnexin and NE protein emerin), and Fig. 7 (ER protein LEM4), as detailed in previous major point 3. These do not

indicate appreciable UPR. It may also be worth mentioning that Tsai *et al.* (2016) reported that LBR degradation appears not to follow the generalized endoplasmic-reticulum-associated degradation (ERAD) pathway linked to the UPR, but an alternative degradation pathway, occurring at the NE and accumulating LBR degradation products inside the nucleus (a phenotype we have never observed in our silencing experiments, consistent with lack of UPR).

In regard to the lipid profile and to respond to the Reviewer's request:

(a) **we now provide the full lipidomic analysis in a new supplementary figure (Suppl. Table S2)** so that any interested reader can have access to the data of all lipid species. To the question of the Reviewer about phospholipids, we observe relative increase in aPC (phosphatidylcholine) and decrease in aPE (phosphoethanolamine) in TMEM147-silenced cells. Other phospholipids are unchanged.

(b) we have now conducted **a new multivariate analysis** of the full lipidomics data. A principal component analysis (PCA) of the lipidome indicated that TMEM147-silenced cells separated from the control-silenced and untreated cells along the first principal component. Using the loadings plot, we identified that cholesterol and cholesteryl esters (CE) were amongst the most significant features that drive this separation. This clearly confirms, as per our original manuscript, that cholesterol and cholesteryl esters are important lipids, discriminating TMEM147-silenced from control-silenced and untreated cells. Furthermore, regarding the individual CE species, it reconfirms our observation that saturated and short lipid species are significantly reduced while poly-unsaturated long species are significantly upregulated. Overall, total CEs are downregulated. We have replaced the older Suppl. Fig. S5 with the new expanded figure to include this more elaborate analysis (**renamed Suppl. Fig. S6**).

In conclusion, for clarity we provide the complete lipid profile (Suppl. Table S2). However, we would like to refrain from providing a still wider perspective on lipid metabolism in the manuscript, which is outside the scope of the current paper and a full new project in itself. We believe that our findings on cholesterol metabolism as the focus of the current manuscript are well supported.

Minor points

1) Page 9, the authors *state "TMEM147-GFP displayed the same distribution as the Flag- tagged version"*. They actually do not look the same. Comparing Fig. 1 A1 and B1, the GFP- tagged TMEM147 does not effectively localize to the nuclear envelope, and does not co- localize with Lap2beta (INM marker) in Fig.1 D3. Does the GFP tag affect the distribution of TMEM147?

The GFP-tagged TMEM147 does localize to the NE as well as the rest of the ER, as we observed time and time again (as, for example, also seen in Suppl. Fig. S1 panel C1/C3 and Fig. 2, panel D2). In response to the reviewer's comment, **we have now replaced panels B1 and B2 in Fig. 1** with new images of TMEM147-GFP (and at higher magnification), in which labeling of the NE is clearer than in our previous example and as obvious as with the FLAG- tagged version. We thank the Reviewer for prompting this change to more representative examples.

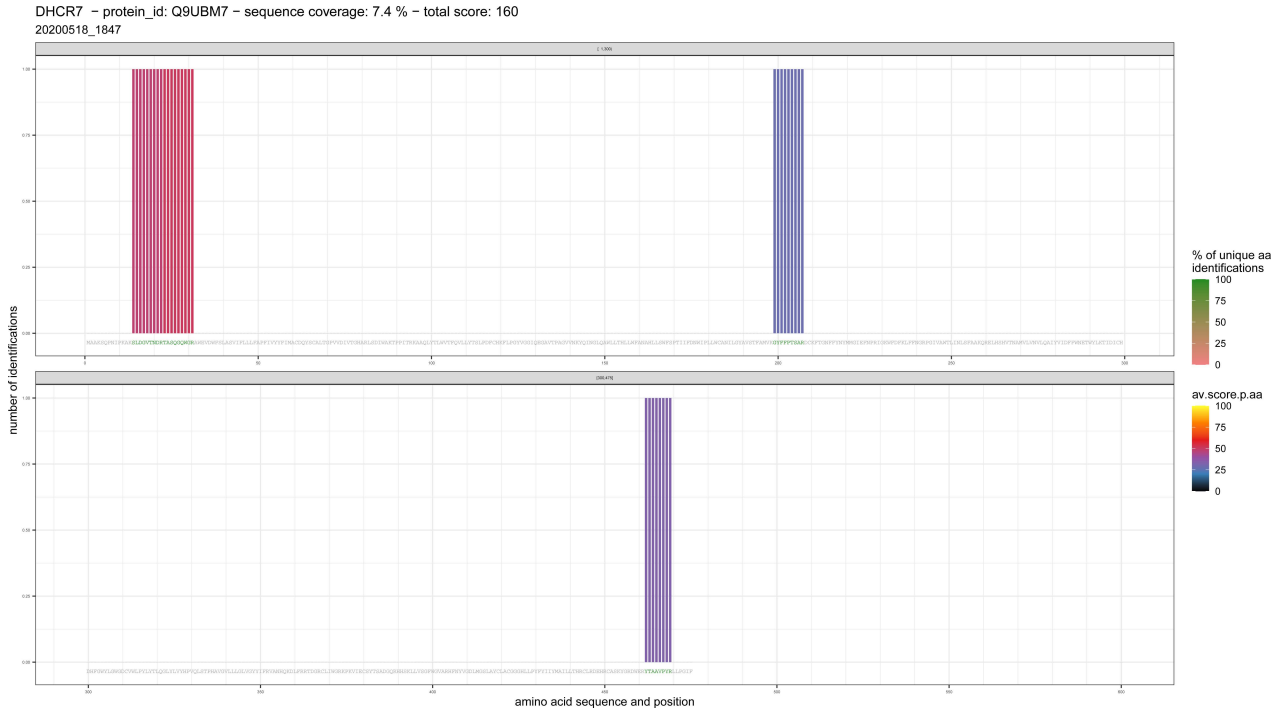
In panel D3, the co-localisation with Lap2β is seen as a yellow rim at the nuclear periphery.

2) Fig. 6, Panel B5. The quality of the TMEM147 blot is suboptimal, this experiment should be repeated and a specificity control included (e.g. using an antibody against an irrelevant polytopic protein).

We are sorry for the suboptimal part of the blot. We have **repeated the experiment** and now provide a good image in revised Fig. 6B5. As explained in our response to the last point of Reviewer 1, we also provide **additional supportive data** in new panel C of Suppl. Fig. S3, showing that the other N-terminal construct, HeLa-LBR238-GFP, was also unable to bind the native TMEM147, as was the GFP-only bait, used as a negative control (Results, p.14). The fact that both N-terminal constructs show no interaction (in contrast to the full-length and the C-terminal construct) serves as appropriate (internal) specificity controls to indicate that these results are not due to random artefactual interactions of polytonic membrane proteins.

3) The authors state that TMEM147 and DHCR7 interact. How exactly was the IP/MS experiment performed? The IP looks far from clean (not unusual for detergent extracts/membrane protein IPs), normally 100s of proteins would be detected even in an excised gel band with any contemporary standard MS setup. Therefore, the sequence coverage of DHCR7 should be shown, along with a rank list of identified proteins. The authors should confirm this IP via IP/Western blotting and importantly, using suitable controls (GFP is not a suitable control, cf. major point 2). Alternatively, all DHCR7 data could simply be omitted.

We apologise for the confusing manner the IP/MS experiment was presented, this was clearly not effective. This point was also brought up by Reviewer 1 (point 3). As mentioned already, **we have now conducted a brand-new IP/MS analysis, which we show in the new Suppl. Fig. S4 and incorporated in the Results (p.14).** Please, kindly refer to our full explanations on the experiments and its results, in our answer to Reviewer 1 point 3 (p.2). We note that an ER polytonic protein, TMEM129, was used for comparison/validation in this analysis, as suggested by the Reviewer.
In addition to the data shown in the manuscript and because we were close to the limit for manuscript/figure length, we provide the specific information the Reviewer is requesting on DHCR7 here, for perusal (sequence coverage 7.4%).



3) In Fig, 7E (and corresponding text sections) is it really absorbance that is monitored or rather fluorescence?

Thank you for spotting this oversight. It is fluorescence and this mistake **has been corrected** both in revised Fig. 7 and in the associated legend text (p.31+32).

4) In the discussion (2nd paragraph), the author state that changes in cholesterol metabolism imposed by TMEM147 depletion are responsible for the observed toxicity. This interpretation seems unlikely since HeLa LBR KO cells grow normally in regular medium unless they are shifted to cholesterol-restrictive conditions. Even then, the growth defects can be rescued by addition of LDL or cholesterol (cf. Tsai et al., PMID: 27336722). It seems equally likely that other defects account for the observed toxicity. This could be discussed or addressed experimentally.

Similar questions were also raised by Reviewer 1. As explained in our response to point 2 by Reviewer 1, we were prompted to thus conduct an additional experiment to address these comments (Fig. 7 F and G) and so we kindly refer to our detailed description of our new results in this response as it also addresses these points by Reviewer 3.

5) Page 13, *"At the same time, total levels of cholesteryl esters (CE) were greatly reduced, by 68% (5.80±1.68 mol% in TMEM147-silenced cells vs. 9.74±1.83 in control"*. 9.74% to 5.8% is not equal to "reduced by 68%".

We apologise for the oversight **and have now corrected the passage to**:
"At the same time, total levels of cholesteryl esters (CE) were greatly reduced, by 40.5% (5.80±1.68 mol% in TMEM147-silenced cells vs. 9.74±1.83 in control".

Additional Editor's request:

(a) We **have now abbreviated the title** to comply with the Editor's request for no more than 120 characters.

(b) Finally, we have made small **formatting changes** in the manuscript (including reference citation and list) to fully comply with the stylistic requirements of the Journal and have taken the opportunity to screen for and correct any remaining typing mistakes.

References quoted in this letter

Bartoli, D., Piobbico, D., Bellet, M. M., Bennati, A. M., Roberti, R., Della Fazia, M.A. and Servillo, G. (2016). Impaired cell proliferation in regenerating liver of 3 beta-hydroxysterol Delta14-reductase (TM7SF2) knock-out mice. *Cell Cycle,* **15,** 2164-2173.

Bennati, A.M., Schiavoni, G., Franken, S., Piobbico, D., Della Fazia, M.A., Caruso, D., De Fabiani, E., Benedetti, L., Cusella De Angelis, M.G., Gieselmann, V., et al. (2008). Disruption of the gene encoding 3beta-hydroxysterol Delta-reductase (Tm7sf2) in mice does not impair cholesterol biosynthesis. *FEBS J,* **275,** 5034-47.

Capell-Hattam, I.M., Sharpe, L.J., Qian, L., Hart-Smith, G., Prabhu, A.V. and Brown, A.J (2020) The cholesterogenic enzymes DHCR14 and LBR are differentially regulated transcriptionally and post-translationally. *J. Biol. Chem*, **295.** 2850-2865

Clayton, P., Fischer, B., Mann, A., Mansour, S., Rossier, E., Veen, M., Lang, C., Baasanjav, S., et al. (2010) Mutations causing Greenberg dysplasia but not Pelger anomaly uncouple enzymatic from structural functions of a nuclear membrane protein. *Nucleus*. **1**, 354–366.

Dettmer, U., Kuhn, P.H., Abou-Ajram, C., Lichtenthaler, S.F., Kruger, M., Kremmer, E., Haass, C. and Haffner, C. (2010). Transmembrane protein 147 (TMEM147) is a novel component of the Nicalin-NOMO protein complex. *J Biol Chem,* **285,** 26174-81.

Holmer, L., Pezhman, A. and Worman, H.J. (1998) The human lamin B receptor/sterol reductase multigene family. *Genomics*. **54**, 469– 476.

Frolov, A., Petrescu, A., Atshaves, B.P., So, P.T.C., Gratton, E., Serrero, G. and Schroeder, F. (2000) High density lipoprotein mediated cholesterol uptake and targeting to lipid droplets in intact L-cell fibroblasts. *J. Biol. Chem.*, **275**, 12769-12780

Huang, H., McIntosh, A.L., Landrock, K.K., Landrock, D., Storey, S.M., Martin, G.G., Gupta, S., Atshaves, B.P., Kier, A.B. and Schroeder, F. (2015) Human FABP1 T94A variant enhances cholesterol uptake. *Biochimica et Biophysica Acta (BBA)* - Molecular and Cell Biology of Lipids, Volume 1851, Issue 7, Pages 946-955.

Li, X., Roberti, R. and Blobel, G. (2015). Structure of an integral membrane sterol reductase from Methylomicrobium alcaliphilum. *Nature,* **517,** 104-7.

Mattout, A., Cabianca, D.S. and Gasser, S.M. (2015). Chromatin states and nuclear organization in development-a view from the nuclear lamina. *Genome Biol.,* **16(1),** 174.

Nikolakaki, E., Mylonis, I. and Giannakouros, T. (2017). Lamin B Receptor: Interplay between

Structure, Function and Localization. *Cells,* **6**.

Olins, A.L., Rhodes, G., Welch, D.B., Zwerger, M. and Olins, D.E. (2010). Lamin B receptor: multi-tasking at the nuclear envelope. *Nucleus,* **1,** 53-70.

Prabhu, A.V., Sharpe, L.J. and Brown, A.J. (2014). The sterol-based transcriptional control of human 7-dehydrocholesterol reductase (DHCR7): Evidence of a cooperative regulatory program in cholesterol synthesis. *Biochim Biophys Acta* **1842,** 1431-1439.

Prabhu, A.V., Luu, W., Sharpe, L.J. and Brown, A.J. (2016). Cholesterol-mediated Degradation of 7-Dehydrocholesterol Reductase Switches the Balance from Cholesterol to Vitamin D Synthesis. *J Biol Chem.* **291,** 8363-8373.

Rosemond, E., Rossi, M., Mcmillin, S.M., Scarselli, M., Donaldson, J.G. and Wess, J. (2011). Regulation of M(3) muscarinic receptor expression and function by transmembrane protein 147. *Mol Pharmacol,* **79,** 251-61.

Solovei, I., Wang, A.S., Thanisch, K., Schmidt, C.S., Krebs, S., Zwerger, M., et al. (2013). LBR and lamin A/C sequentially tether peripheral heterochromatin and inversely regulate differentiation. *Cell,* **152,** 584–98.

Tsai, P.L., Zhao, C., Turner, E. and Schlieker, C. (2016). The Lamin B receptor is essential for cholesterol synthesis and perturbed by disease-causing mutations. *Elife,* **5**.

Ye, Q. and Worman, HJ. (1994). Primary structure analysis and lamin B and DNA binding of human LBR, an integral protein of the nuclear envelope inner membrane. *J Biol Chem.* **269,** 11306–11311.

Zwerger, M., Herrmann, H., Gaines, P., Olins, A.L. and Olins, D.E. (2008). Granulocytic nuclear differentiation of lamin B receptor- deficient mouse EPRO cells. *Exp Hematol,* **36,** 977-87.

Second decision letter

MS ID#: JOCES/2020/245357

MS TITLE: ER protein TMEM147 interacts with Lamin B Receptor, regulates its levels/localization and affects cholesterol synthesis

AUTHORS: Andri Christodoulou, Giannis Maimaris, Andri Makrigiorgi, Evelina Charidemou, Christian Luechtenborg, Antonis Ververis, Renos Georgiou, Carsten Werner Lederer, Christof Haffner, Britta Bruegger, and Niovi Santama
ARTICLE TYPE: Research Article

I have sent your revised manuscript to the three original reviewers.

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, reviewer #1 finds that you have satisfactorily addressed his/her comments and recommends publication. However, reviewers #2 and #3 still have some minor and major issues that you will need to address before submitting your final manuscript for publication. I trust that you will be able to properly deal with them and look forward to receiving a further revision of your paper.

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.

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 well to my previous critiques and questions and made the necessary changes to the manuscript. To my opinion they also addressed most of the concerns raised by the other two Reviewers. This is a well-written manuscript providing evidence that TMEM147 regulates cholesterol homeostasis in cells through the modulation of LBR and DHCR levels. My suggestion is that it should be accepted for publication.

Comments for the author

The authors have responded well to my previous critiques and questions and made the necessary changes to the manuscript. To my opinion they also addressed most of the concerns raised by the other two Reviewers, therefore my suggestion is that the manuscript should be accepted for publication.

Reviewer 2

Advance summary and potential significance to field

Although the authors have attempted to address my concerns, they need to be more precise about claims of effects on cholesterol synthesis when they have not directly measured this parameter.

Comments for the author

Since the authors do not directly measure cholesterol synthesis, the title of the manuscript should be changed to:
“ER protein TMEM147 interacts with Lamin B Receptor, regulates its levels/localization and affects cholesterol homeostasis.”
And the subheading on P15 should be changed to: “Silencing of TMEM147 impacts cellular cholesterol homeostasis”

P16, 2nd paragraph: “CE levels, as storage forms of cholesterol, give a measure of cellular cholesterol biosynthesis”
This statement should be supported by a reference directly showing this, or rephrased.

Reviewer 3

Advance summary and potential significance to field

Overall, the manuscript improved over the original version in several regards. The data clearly show that TMEM147 knockdown result in a decrease of LBR and DHCR7 on the protein level, and also that LBR re-localizes partly in the ER. The reviewer’s concerns to include other ER and nuclear markers

were adequately addressed. Furthermore, the requested lipid profiling was performed. Overall, it's an interesting and novel finding that knockdown TMEM147 (a poorly characterized protein) causes the decrease of enzymes responsible for cholesterol synthesis in conjunction with a cellular phenotype and therefore, publication in JCS is recommended. Below are some final points for clarification that could be addressed experimentally, or alternatively, could be addressed by amending the text (i.e. not necessarily requiring additional experiments)
prior to publication:

Comments for the author

Below are some final points for clarification that could be addressed experimentally, or alternatively, could be addressed by amending the text (i.e. not necessarily requiring additional experiments) prior to publication:

1) This reviewer is (still) not entirely convinced that the observed cellular toxicity upon TMEM147 knockdown can be solely explained by defects in cholesterol biosynthesis, as phenotypic changes are observed even in the absence of cholesterol restriction where cells would normally acquire cholesterol via endocytic uptake from media and the entire biosynthetic pathway is essentially turned off. Even a dual effect due to the loss of LBR and DHCR7's enzymatic activities cannot account for those effects unless under conditions of cholesterol starvation. However, it is also evident that cholesterol addition rescues some of the growth phenotypes to a certain degree. All things considered, an alternate, not mutually exclusive interpretation is that sth additive might be going on. Perhaps it would be a good idea to briefly address this point in the discussion, e.g. through inclusion of a cautionary note stating that additional effects cannot be formally excluded at this point.

2) Our request to use an irrelevant polytopic protein (rather than GFP) as control was not appropriately addressed. Especially the interaction reported in Fig. 5 and 6 B5 would be more convincing with such a control in stand-alone experiments, especially since the interaction appears to be quite weak. Admittedly many papers, including those published in JCS, are published without this control, however this reviewer remains of the opinion that this interaction would be much more convincing with a suitable control. I'm not doubting that this interaction is real but please note that in our hands, several "interactions" reported in the literature using analogous co-IPs w/o adequate controls turned out to be false-positives which can be attributed to mixed detergent micelles. It's a notorious problem with polytopic membrane proteins.

3) In the opinion of this reviewer it would be better to use an a - b - c -d (etc) scheme to label the panels in e.g. Fig. 6.

3) On a side note, neither Emerin nor Calnexin are suitable UPR markers, and certainly not a standard in the field. It would be better to monitor BiP levels or XBP-1 splicing, both of which are easy to do using commercially available reagents. However, in this reviewers' opinion these experiments are not essential and I don't want to suggest add-on experiments in a second review recycle. Alternatively, the authors could simply remove the conclusion that UPR is not induced (though admittedly they don't make a major point out of this).

Second revision

Author response to reviewers' comments

Point-by Point Response

We would like to thank all the Reviewers for their previous constructive criticism, which resulted in a much-improved manuscript. We would like to address their remaining queries (blue font), and hope that our second revision will meet approval for publication. New corrections are marked red in the submitted manuscript revision.

Below are our responses to the comments by the Reviewers.

Comments by Reviewer 1

Reviewer 1 Comments for the Author:

The authors have responded well to my previous critiques and questions and made the necessary changes to the manuscript. To my opinion they also addressed most of the concerns raised by the other two Reviewers, therefore my suggestion is that the manuscript should be accepted for publication.

We are grateful and thankful to the Reviewer for the positive appraisal of our revision and suggestion for manuscript publication.

Comments by Reviewer 2

Reviewer 2 Comments for the Author:
Since the authors do not directly measure cholesterol synthesis, the title of the manuscript should be changed to:
"ER protein TMEM147 interacts with Lamin B Receptor, regulates its levels/localization and affects cholesterol homeostasis."
And the subheading on P15 should be changed to: "Silencing of TMEM147 impacts cellular cholesterol homeostasis"

We **have made both changes** that the Reviewer is requesting to the title and subheading (p.14).

P16, 2nd paragraph: "CE levels, as storage forms of cholesterol, give a measure of cellular cholesterol biosynthesis" This statement should be supported by a reference directly showing this, or rephrased.

It is understood that cellular cholesterol levels can regulate the pathway of cellular cholesterol synthesis and that high cholesterol levels allosterically activate ACAT1&2, the ER enzymes responsible for the esterification of cholesterol to cholesteryl esters (CEs). Thus, the levels of intracellular free (non-plasma membrane bound) cholesterol available/arriving at the ER and the formation of CEs are dynamically interlinked and this is what we were trying to say here.

As requested by the Reviewer, **we have rephrased this sentence** more accurately to *"CEs, as the intracellular storage forms of excess cholesterol, are of central importance to cholesterol homeostasis and their formation is a measure of the availability of cellular free cholesterol (Luo et al., 2020)"* (p.15), to summarize current understanding of the dynamic link between the two types of metabolite, which we hope will be satisfactory.

Reference quoted (and added to reference list): Luo J, Yang H, Song BL. (2020) Mechanisms and regulation of cholesterol homeostasis. *Nat Rev Mol Cell Biol*. 2020;21(4):225-245. doi:10.1038/s41580-019-0190-7

Comments by Reviewer 3

Reviewer 3 Advance Summary and Potential Significance to Field:

Overall, the manuscript improved over the original version in several regards. The data clearly show that TMEM147 knockdown result in a decrease of LBR and DHCR7 on the protein level, and also that LBR re-localizes partly in the ER. The reviewer's concerns to include other ER and nuclear markers were adequately addressed. Furthermore, the requested lipid profiling was performed. Overall, it's an interesting and novel finding that knockdown TMEM147 (a poorly characterized protein) causes the decrease of enzymes responsible for cholesterol synthesis in conjunction with a cellular phenotype and therefore, publication in JCS is recommended.

We are thankful for the positive appraisal and publication recommendation by the Reviewer.

Reviewer 3 Comments for the Author:

Below are some final points for clarification that could be addressed experimentally, or alternatively, could be addressed by amending the text (i.e. not necessarily requiring additional experiments) prior to publication:

1) This reviewer is (still) not entirely convinced that the observed cellular toxicity upon TMEM147 knockdown can be solely explained by defects in cholesterol biosynthesis, as phenotypic changes are observed even in the absence of cholesterol restriction where cells would normally acquire cholesterol via endocytic uptake from media and the entire biosynthetic pathway is essentially turned off. Even a dual effect due to the loss of LBR and DHCR7's enzymatic activities cannot account for those effects unless under conditions of cholesterol starvation. However, it is also evident that cholesterol addition rescues some of the growth phenotypes to a certain degree. All things considered, an alternate, not mutually exclusive interpretation is that sth additive might be going on. Perhaps it would be a good idea to briefly address this point in the discussion, e.g. through inclusion of a cautionary note stating that additional effects cannot be formally excluded at this point.

We have now **made the exact cautionary note** the Reviewer is proposing in the Discussion: *"Addition of exogenous cholesterol increased cell viability but we cannot formally exclude additive detrimental effects of TMEM147 silencing, contributing to increased cell death rates."* (p.17).

2) Our request to use an irrelevant polytopic protein (rather than GFP) as control was not appropriately addressed. Especially the interaction reported in Fig. 5 and 6 B5 would be more convincing with such a control in stand-alone experiments, especially since the interaction appears to be quite weak. Admittedly, many papers, including those published in JCS, are published without this control, however this reviewer remains of the opinion that this interaction would be much more convincing with a suitable control. I'm not doubting that this interaction is real but please note that in our hands, several "interactions" reported in the literature using analogous co-IPs w/o adequate controls turned out to be false-positives which can be attributed to mixed detergent micelles. It's a notorious problem with polytopic membrane proteins.

We are in agreement with the Reviewer's general point and have been very cautious ourselves in evaluating these interactions using different lines of experiments. We remain convinced of the interactions between TMEM147 and LBR and DHCR7, as well as the significance of the C-terminal domain of LBR in its physical interaction with TMEM147.

(1) Most significantly, both LBR and DHCR7 not only came up within top hits in our proteomics analysis (Fig. S4) where we had used the polytopic protein TMEM129 in parallel (we certainly do not exclude that some of the other proteins identified, but not further tested in this manuscript, may turn out to be non-specific interactions) or in specific pairwise co-IP queries (for LBR Fig. 5+6), but, importantly, both sterol reductases displayed functional interactions with TMEM147 upon TMEM147 silencing, and co-ordinate transcriptional responses upon serum starvation combined with TMEM147 silencing. Taken together, and with the corroboration of lipidomic analysis on the effect of TMEM147 silencing on cholesterol/CEs levels, these results make a strong point overall, in our opinion, about the validity of these interactions.

(2) For the experiments shown in Fig. 6B5, we would like to say that we are not surprised that the biochemical signals of interaction are not stronger, given that these assays rely on transient transfections and that the proteins involved are all polytopic transmembrane proteins (not the easiest types of proteins to transfect and localize effectively). What is important to emphasise here is that by repeating the experiments many times, we consistently observed that:
(a) the interaction of TMEM147 with the C-terminal LBR construct (lane f) is as strong as that observed with the full-length LBR (lane i),
(b) experiments with two different LBR N-terminal constructs (LBR^{272}-GFP and LBR^{238}-GFP, the latter having been extensively used in LBR research) (Fig. 6B5 and S3C), have shown lack of interaction with TMEM147. We should stress that these LBR fusion proteins are polytopic proteins themselves, albeit not additional, completely unrelated, controls.
(c) GFP-only (not a membrane-specific control but an acceptable generic control for lack of non-

specific protein interaction) gave no background, attesting to, at least, our careful/clean experimentation.

In conclusion, we are happy that the Reviewer does not question the authenticity of these interactions which we believe we substantiated with experiments using different approaches, all of which combined serve to affirm their specificity.

3) In the opinion of this reviewer it would be better to use an a - b - c -d (etc) scheme to label the panels in e.g. Fig. 6.

This would be a straightforward revision for us to make, but, when comparisons need to be made across related panels we find the current format of pair labeling (A1, A2 etc) more intuitive to the reader, and not contrary to the style of published manuscripts in the JCS. We therefore prefer not to introduce this change if the Reviewer does not mind, but to leave it at the discretion of the Editor. If required, we will easily make the suggested change to formatting.

3) On a side note, neither Emerin nor Calnexin are suitable UPR markers, and certainly not a standard in the field. It would be better to monitor BiP levels or XBP-1 splicing, both of which are easy to do using commercially available reagents. However, in this reviewers' opinion these experiments are not essential and I don't want to suggest add-on experiments in a second review recycle. Alternatively, the authors could simply remove the conclusion that UPR is not induced (though admittedly they don't make a major point out of this).

We had included emerin, an INM integral protein (like LBR), as a negative control INM marker in Fig. 3 and not in reference to UPR. For UPR markers, we had to work with antibodies that were already available in the laboratory as it was impossible to order new antibodies (or anything else) at the lockdown period, during which all of the revision work was carried out (we received the reviews on March 13th and submitted the revision at deadline, on June 6th, a few days after reopening in Cyprus). So, we would like to thank the Reviewer for giving us the option to address this in the text. As suggested, we have now **removed reference to ER stress** both in the Results section and in the legend of Fig. 3 (p.11+p.28).

Third decision letter

MS ID#: JOCES/2020/245357

MS TITLE: ER protein TMEM147 interacts with Lamin B Receptor, regulates its levels/localization and affects cellular cholesterol

AUTHORS: Andri Christodoulou, Giannis Maimaris, Andri Makrigiorgi, Evelina Charidemou, Christian Luechtenborg, Antonis Ververis, Renos Georgiou, Carsten Werner Lederer, Christof Haffner, Britta Bruegger, and Niovi Santama
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.