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## Seipin regulates ER-lipid droplet contacts and cargo delivery

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### Review timeline:

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### Transaction Report:

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

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 1st Editorial Decision

 29 August 2016
 

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Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see, the referees appreciate your work. However, they also think that some of the data need a better analysis and that additional insight is required. They all provide clear and constructive reports, and addressing the issues raised seems straight forward. Given the referees' positive recommendations, I would thus like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. Importantly,

- the imaging data need to be better quantified (see reports from all three referees).
- a potential tethering function for seipin needs to be substantiated by additional data and/or better discussed (referee #1, point 3 and referee #2, point 1)
- a potential role of DGAT2 for the observed defect in neutral lipid synthesis needs to be addressed (referee #1, point 6)
- controls of expression levels need to be provided (referee #1, point 7)
- the quality of some of the presented imaging data needs to be improved (referee #2, point 2)
- the known literature needs to be better integrated into the present work and the relevance for pathophysiologic states needs to be better discussed and analyzed (referee #3, point 1 and 2)
- the temporal analysis of LDs needs to be extended (referee #3, point 4.1, 4.3 and 7)

## REFeree REPORTS

### Referee #1:

Seipin is an evolutionarily conserved protein with a central role in lipid droplet (LD) function. In this manuscript Ikonen and co-workers investigate the function of Seipin in human cells. It is described that ablation of Seipin in human A431 cells (using CRISPR/Cas9 system) or a loss of function mutation in BSCL2-patient derived cells results in altered LD morphology and dynamics. These defects could be corrected by re-expression of GFP-Seipin, which was enriched at ER-LD contact sites. Electron microscopy showed that these ER-LD contacts were aberrant in Seipin mutant cells. In agreement with these alterations, the authors report that the targeting of ER membrane proteins to LDs is defective in Seipin KO cells. The defect was particularly strong for ACSL while HPos (an artificial LD targeting reporter) appeared to target LDs at an initial phase but not at later stages. This bi-modal behaviour was also observed for traceable fatty acid-analogs. The data is of very high quality and largely convincing. Furthermore this work confirms previous studies in budding yeast (from Carvalho and Goodman's groups) that showed that Seipin by stabilizing ER-LD contacts facilitates the incorporation of protein and lipid cargo into growing LDs. Overall, the a unifying role of Seipin in LD biogenesis together with the observation that aberrant ER-LD contacts might be relevant for the pathophysiology of seipin-derived lipodystrophies are important additions that should warrant the interest of the EMBO Journal. However, before publication there are a number of issues listed below that should be addressed.

1- The data presented here suggest a biphasic process in LD biogenesis, with the initial phase being less dependent on Seipin function while later stages require Seipin function for the stabilization of functional ER contacts. Were there any structural differences in the ER-LD contacts in WT cells that may explain the different Seipin requirement at early and late stages of LD formation?

2- It is described that in WT cells LDs exhibit a close physical relationship with the ER with Seipin stably localizing at the ER-LD junctions. The electron microscopy data and reconstructions shown are of high quality so the authors should make an effort to describe better the ER-LD contacts. In the images (and reconstruction profiles) are bridges or membrane continuities seen? If yes, in which fraction of LDs? Does this change in seipin KO cells?

3- Still related to the previous point, the authors see that in Seipin KO cells a small fraction of LDs (~10%) appears detached from the ER thus proposing that seipin tethers LDs to the ER. In this reviewer's opinion, the data shown in the paper is not sufficient to support this conclusion (and particularly to use it in the title!). Are the detached LDs different in other parameter (for example size). Does the fraction of detached LDs changes over time (upon OA addition)?

4- In seipin KO cells the ER appeared occasionally expanded in the vicinity of LDs (Fig 3A). In Fig 2 F panels I and II (wt cells) appear different from panel III (SKO). Are these representative images? Are the ER morphological alterations restricted to the ER-LD contacts or other morphological changes are seen in seipin KO cells?

5- The STORM measurements found differences in the diameter of WT and A212P GFP structures. Did the differences reflect the association/contact with LDs or only structures in contact with LD were selected for the measurements?

6- The authors detect a defect in neutral lipid synthesis during later stages of LD formation, which according to the literature should depend on DGAT2. Did the authors checked whether DGAT2 localizes in the KO cells?

7- The conclusions about the differences in localization of wt and A212P GFP-Seipin would be strengthened by showing that they are not simply due to different levels of expression. Also, how does the levels of these GFP fusion compare to endogenous Seipin?

8- In Supplementary Fig 5A, is it expected that delipidated cells still accumulate such high amounts of neutral lipids?

## Referee #2:

This paper probes the functional role of Seipin at ER-LD contact sites in animal cells. Seipin is an integral ER membrane protein. Nice imaging data shown here localize Seipin to dynamic ER puncta. Nearly all LDs track together with a Seipin punctae at a MCS. They generate knockout Seipin cells (SKO) to test the effect of depletion on LD maturation, ER-LD tethering, and trafficking of lipids and proteins between ER-LD MCSs. SKO cells have smaller more abundant LDs, variable tethering, and can traffic lipids and proteins between the ER and small but not larger LDs. Suggests that LD size in SKO cells could be blocked because components stop trafficking from ER to LDs. Nice control, cytoplasmic proteins can still traffic to LD in SKO.

This story does a good job characterizing the phenotype of Seipin in animal cells. Not a new protein, Seipin has been linked to ER-LD contact functions before in yeast, where KO gives the opposite phenotype. Data shown here does a convincing job to demonstrate that Seipin KOs have reduced protein/lipid trafficking at ER-LDs. However some concerns must be addressed:

- 1) One big issue is that I don't agree with the general conclusion that Seipin is a tether because on average SKO cells actually have increased ER-LD contact (just a minor population has lost contact). That is not the phenotype of a tether.
- 2) The quality of the EM data in F1G and Fig 4G is not sufficient for quantification. Membrane bound organelles, like LDs, should have smooth not angular lines, membranes appear smashed against each other by fixation, and how are they identifying the opposing membrane as ER when it is too wide to be ER and not ribosome bound?
- 3) LDs in SKOs look like background staining in F1A.

## Referee #3:

In this manuscript, the authors characterize the role of WT Seipin in lipid droplet (LD) biogenesis and investigate how the absence of Seipin (SKO) affects LD biology in A431 cells. They aim to link it to a human pathological mutation in patients harboring a mutation in the Seipin gene (A212P) and they also use patient fibroblasts.

In summary, in the absence of Seipin, newly formed LDs are smaller, less associated with the ER and move faster. Seipin localizes to the ER/LD contact site. In the absence of Seipin, ACSL3 is not recruited to forming LDs (whereas the recruitment of probe Hpo and the lipid Bodipy are not affected). Conversely, the absence of Seipin affects the recruitment of Hop and Bodipy in "older"/formed LDs.

The paper is data rich and represents a lot of hard work. The experiments are well executed. The ms is primarily microscopy based. In particular, the authors use a number of very fancy/new light and electron microscopy based imaging techniques (although not all of them always completely justified).

The key protocol is to delipidate A431 cells (WT or CRISPR Seipin KO, SKO) overnight and feed them with large amount of oleic acid to stimulate acute lipid droplet biogenesis.

1) The results are interesting and new, although with the publication of the yeast paper on the Seipin homolog last year, I would insist to have a more open comparison, what is similar and which new concepts this study brings forward. I would insist on this.

2) The main issue I have with these data is that the authors aim to link their data to a pathological situation.

-The cells used for these studies is not relevant to fat metabolism. A431 cells are of epidermoid carcinoma origin. At least they could have performed some experiments in adipocytes.

-Their experimental setting informs an acute situation, not a long term steady state as it is the case in patients. The simple fact that SKO clones were made and are viable means that its role in these cells is not as critical as in patients who are quite sick.

-Patients harbor a mutated Seipin whereas their experimental system lacks Seipin altogether. It is

actually possible that the mutated protein is more toxic than the absence of the WT protein.

Altogether, I have a hard time understanding how the presented data on acute LD biogenesis explain the phenotype of 0% fat in patients?

Do these fast non ER associated LDs fail to get neutral lipid after neogenesis?

Can Seipin have two roles, one in LD biogenesis and another on formed/older LDs to recruit lipids, perhaps by keeping the tethering to the ER?

Data suggest that it might be the case (as Hpo and BDY C12 are not recruited to formed/older LDs in the absence of Seipin).

Therefore an analysis on older/steady state LDs would be necessary.

#### Specific comments

3) In SKO, newly synthesized lipid droplets (LD) are numerous but smaller and moving faster, less directionally than WT. Furthermore, a larger subset of LDs are not associated with ER and those are the ones that move fast.

In my view, it is essential to quantify all these facts.

3.1: Smaller LD in SKO: How much smaller? Is there an increase/decrease in total LD volume in WT versus SKO?

Is their composition different in lipids? In proteins? At steady state?

3.2: What is the LD pattern in cells that are not challenged by delipidation and OA feeding?? Is there a difference between WT and SKO? What is this?

3.3: Mobility is assessed during 6 seconds. This is not very long and reflects the mobility of newly formed LDs. What is the mobility of older LDs? Is there a difference between WT and SKO?

3.4: How many WT LDs dissociate from ER after biogenesis. Is the SKO phenotype not only an accelerated kinetics of detachment? Is it possible that Seipin stabilizes the contact?

4) WT Seipin localizes to LD. Nearly each LD is decorated by one spot of Seipin. The localization of the mutant protein with respect to LD is not investigated but it appears more ER.

4.1: Also not investigated is whether this Seipin dot on LDs is stable over time? As mentioned above, the study looks at the acute formation of LDs but it would be important (with respect to the clinical situation) to show whether older LDs contain Seipin as well, how long Seipin remains on LDs, whether it changes, how, etc.

4.2: Using STORM, The authors show that the structure decorated by WT Seipin is 15% larger than this decorated by mutant Seipin. This is an unnecessary piece of information and looks more like a technological display. Please place this in Suppl figure.

4.3: Last, by EM, WT Seipin is confirmed to localize at LD/ER contact whereas mutant Seipin appears restricted to the ER as suggested by the IF analysis.

Same comment as above regarding older LDs.

5) The LD/ER contacts are then investigated in more details using 2 different EM methods.

The first shows that in a WT situation, LDs contact the ER at discrete sites whereas in SKO, more and smaller LDs are away from ER, thus confirming the light microscopy data.

The second shows that in WT, the LD/ER contacts are refined whereas in SKO, when LDs are in contact with ER, the contact area is larger. Those LDs appear wrapped up. Thus the authors reach the same conclusion as in yeast (Grippa et al 2015).

However, they also found examples of flimsy contacts, perhaps from LDs that are not tethered properly and leave the ER vicinity.

6) What is the relevance of the defects in LD/ER contacts?

The authors investigate the recruitment of ACSL3 to LDs, a protein that is reported to mark

lipogenic ER subdomains and nascent LDs.

- In the absence of Seipin ACSL3 is not recruited to LDs that are here mark by protein ADRP (tagged).

-Using an elegant heterogenous cell fusion, this recruitment is shown to be generated during LD biogenesis. Seipin needs to be present during LD formation to allow ACSL3 recruitment to LDs.

-Endogenous ACSL3 behaves the same as tagged ACSL3. EM reveals that ACSL3 decorates the entire surface of LDs in WT cells whereas it remains at contact sites in SKO and does not appear to diffuse in the plane of the LD monolayer.

This figure (Fig.4) is very oddly presented. I suggest to present data on the endogenous ACSL3 first moving to the tagged version data (A, B) to the suppl, especially because the introduction of yet another marker ADPR makes the ms more complicated and to my view, does not add much. The cell fusion (C) is interesting and informative but after the endogenous data.

7) The authors then re-investigate the ER to LD trafficking via Seipin using a specific probe called Hpo-Cherry and the lipid BDY-C12

In their cases, it is the opposite to ACSL3. Seipin is not required for the targeting of these (artificial) reagents to LDs during the biogenesis of LDs, but Seipin is required for them to reach older/formed LDs.

(as a reminder, for ACSL3, Seipin is required its targeting to LDs during their biogenesis).

This is hard to reconcile with the model proposed by the authors at the end of Figure 6 focusing on ACSL3 in term of LD maturation and acquisition of lipids.

The data on the probes/lipids suggest that Seipin has a second role on older LDs and I would argue that that this role is probably more relevant for the disease.

Again, are those older LDs still associated with the ER? What is the difference between WT and SKO older LDs?

8) The title should mentioned newly formed LDs and the abstract should explain that ACSL3 and Hpo and BDY-C12 behaves differently.

1st Revision - authors' response

10 October 2016

## Response to Editor

Thank you very much for the invitation to submit our revised manuscript "Seipin regulates contacts of newly formed lipid droplets to the endoplasmic reticulum" to The EMBO Journal. I particularly appreciate the helpful discussions with explicit instructions on how to best respond to the reviewers' comments.

I am here providing a short account of the changes made to the manuscript regarding the points you raised. In a separate file, I am providing detailed point-by-point responses to all the reviewers' remarks.

1. The imaging data need to be better quantified (see reports from all three referees).

**The vast majority of our imaging data is now quantified. We have added LD quantifications in both 1 h and 20 h of oleic acid (OA) loading. Most of our data are from early (0.5-1h) LDs, as this is where we observed the earliest defects. The reviewers asked about later time points of LD biogenesis/LDs at steady state, and we have therefore also included data from 20 h of OA loading and from normal growth medium conditions. We have also added quantification of the localization of WT vs. mutant seipin at LD contacts at the light microscopic level (we already had quantification of the immune-EM data in the earlier ms).**

2. A potential tethering function for seipin needs to be substantiated by additional data and/or better discussed (referee #1, point 3 and referee #2, point 1)

**We have now modified the discussion, to more thoroughly discuss a potential tethering, stabilizing vs. other function(s) of seipin. We have also modified the title of the paper to better reflect this.**

3. A potential role of DGAT2 for the observed defect in neutral lipid synthesis needs to be addressed (referee #1, point 6)

**We have added data on DGAT2 localization during LD biogenesis at 20 h of OA loading in wild-type and seipin deficient cells. These data agree with earlier reports on DGAT2 localization in WT cells. We did not observe significant differences between WT and seipin KO cells, which suggests that the localization of DGAT2 is not likely to explain the observed defects in neutral lipid synthesis in seipin KO cells.**

4. Controls of expression levels need to be provided (referee #1, point 7)

**We have added information on the expression levels of seipin in the different systems employed, both at the mRNA and protein level. In addition, we have included new data on seipin tagged with sfGFP at the endogenous locus. These findings are fully in accordance with the conclusions made with the cells with seipin CRISPR KO and WT-Seipin-GFP rescue.**

5. The quality of some of the presented imaging data needs to be improved (referee #2, point 2)

**Referee 2 is requesting better membrane preservation in the immuno-EM images. We tested immunolabeling of thin sections from high pressure frozen, freeze substituted cells, which preserves organelles better. Unfortunately, the anti-GFP antibody no longer works under these conditions and we cannot replace the immuno-EM data. Instead, we have provided images with larger fields of view as supplements, which should help in the orientation and organelle (ER) identification. Please note that referee 1 specifically mentions the EM data to be of high quality, and referee 3 discusses the same immuno-EM data that referee 2 complains about, in a very positive tone, without any criticism on image quality.**

6. The known literature needs to be better integrated into the present work and the relevance for pathophysiologic states needs to be better discussed and analyzed (referee #3, point 1 and 2)

**We have now provided a more detailed discussion on how the available literature is related to the present findings (including the very recent paper from Tobias Walther's and Bob Farese's lab that I mentioned about). In addition, we have included additional biochemical (clickable lipid) data on primary control and seipin mutant patient fibroblasts, demonstrating a similar, delayed defect in neutral lipid synthesis as observed in the A431 cells. This adds to the pathophysiological relevance of our observations.**

7. The temporal analysis of LDs needs to be extended (referee #3, point 4.1, 4.3 and 7)

**We have added data on older (20 h of OA loading) LDs. Based on the rapid and apparently ER independent mobility of the 20 h LDs in seipin KO cells, it seems obvious that the LD phenotype of seipin deficient cells is not restricted to the early time points but that LDs with defective connectivity to the ER are also present at 20 h of OA loading as well as under standard culture conditions (complete medium). We are working on the corresponding 3view EM data, to include these after sending the revised manuscript to the reviewers. At this stage, we have included exemplary thin sections of the 20 h OA loaded LDs in control and seipin KO cells.**

## **Point-by-point responses to the Reviewers' comments for**

### **Salo et al.: Seipin regulates contacts of newly formed lipid droplets to the endoplasmic reticulum**

#### **Referee #1:**

Seipin is an evolutionarily conserved protein with a central role in lipid droplet (LD) function. In this manuscript Ikonen and co-workers investigate the function of Seipin in human cells. It is described that ablation of Seipin in human A431 cells (using CRISPR/Cas9 system) or a loss of function mutation in BSCL2-patient derived cells results in altered LD morphology and dynamics. These defects could be corrected by re-expression of GFP-Seipin, which was enriched at ER-LD contact sites. Electron microscopy showed that these ER-LD contacts were aberrant in Seipin mutant cells. In agreement with these alterations, the authors report that the targeting of ER membrane proteins to LDs is defective in Seipin KO cells. The defect was particularly strong for ACSL while HPos (an artificial LD targeting reporter) appeared to target LDs at an initial phase but not at later stages. This bi-modal behaviour was also observed for traceable fatty acid-analogs. The data is of very high quality and largely convincing. Furthermore this work confirms previous studies in budding yeast (from Carvalho and Goodman's groups) that showed that Seipin by stabilizing ER-LD contacts facilitates the incorporation of protein and lipid cargo into growing LDs. Overall, the a unifying role of Seipin in LD biogenesis together with the observation that aberrant ER-LD contacts might be relevant for the pathophysiology of seipin-derived lipodystrophies are important additions that should warrant the interest of the EMBO Journal. However, before publication there are a number of issues listed below that should be addressed.

**We were delighted about the Reviewer's encouraging words and overall positive assessment of our findings. Below, we address the remaining questions and comments. The major changes in the revised manuscript are highlighted with yellow. Seipin is indeed an enigmatic protein and although we believe that our observations are relevant for the pathophysiology related to seipin dysfunction, we admit that our work is by no means comprehensive and a number of issues remain to be addressed in future studies.**

1- The data presented here suggest a biphasic process in LD biogenesis, with the initial phase being less dependent on Seipin function while later stages require Seipin function for the stabilization of functional ER contacts. Were there any structural differences in the ER-LD contacts in WT cells that may explain

the different Seipin requirement at early and late stages of LD formation?

**This is an interesting and challenging question. The earliest stages of LD formation have been difficult to image by EM. Regarding yeast, Prinz's lab has been able to visualize lenses that nascent LDs form in the ER, apparently before budding from the ER in a process involving FIT proteins (Choudhary et al., J Cell Biol 2015). This may represent the initial phase of LD biogenesis independent of seipin function, and seipin probably acts after this step, stabilizing ER-LD contacts. We – and to our knowledge other groups – have so far not been able to structurally differentiate ER-LD contacts of different ages. This does not mean that such differences might not exist but at least in the cells we studied here (A431 cells and human fibroblasts) there seems to be substantial heterogeneity in the way ER-LD contacts appear at any time point studied, complicating the interpretation. So far, we have not observed obvious time-dependent structural changes in ER-LD contacts that might explain the biphasic seipin dependence.**

2- It is described that in WT cells LDs exhibit a close physical relationship with the ER with Seipin stably localizing at the ER-LD junctions. The electron microscopy data and reconstructions shown are of high quality so the authors should make an effort to describe better the ER-LD contacts. In the images (and reconstruction profiles) are bridges or membrane continuities seen? If yes, in which fraction of LDs? Does this change in seipin KO cells?

**As shown in Figure 3A, some bridges between ER and LD are observed in SKO cells (orange arrowheads). These are present in ~20% of SKO LDs, as observed from thin sections (p. 8 in the revised manuscript). Such bridges were not found in WT cells. As the number of whole LD particles imaged at this resolution is small, we would not like to make too far-reaching conclusions/statistical analysis from this limited material. We did not observe membrane continuities; when the opposing membranes are in very close proximity, the contact area appears thicker, closer to the thickness of two membranes, rather than one shared membrane (as discussed on p. 8). However, it should be noted that due to technical limitations of plastic embedded specimens, we do not exclude the possibility of the existence of some membrane continuities. The only technique that would reliably provide an answer to this question would be cryo-electron tomography of vitreous specimens, currently available only in few labs.**

3- Still related to the previous point, the authors see that in Seipin KO cells a small fraction of LDs (~10%) appears detached from the ER thus proposing that seipin tethers LDs to the ER. In this reviewer's

opinion, the data shown in the paper is not sufficient to support this conclusion (and particularly to use it in the title!). Are the detached LDs different in other parameter (for example size). Does the fraction of detached LDs changes over time (upon OA addition)?

**We agree with the Reviewer, the title was perhaps provocative and we have now changed it. Also, we have modified the discussion, to consider a potential tethering vs. other function(s) of seipin in relation to what other investigators have proposed. When it comes to the detached LDs, they have a relatively uniform size, with a diameter of about 200 nm, suggesting that they might detach from the ER at a certain stage of maturity. In order to address whether the fraction of detached LDs changes over time, we have now included additional data from experiments at a later time point after oleic acid (OA) addition (20 h; Figs 6 and S10). The fraction of small peripheral LDs that move apparently independently of the ER (and may therefore be detached from the ER) is roughly similar, about 20%, at both 1 h and 20 h of OA loading. Thus, our data suggest that this fraction does not change significantly over time. This may be a reflection of continuous formation defective of LDs in seipin deficient cells. The detached LDs may not accumulate if they give rise to additional LD “subtypes” observed more abundantly in later LDs, i.e. aggregated or supersized LDs, and/or if they become degraded over time. In any case, for the present work it is interesting that the novel phenotype found, LDs detached from the ER, is not a transient intermediate but also observed when LDs have been present in cells for longer periods of time.**

4- In seipin KO cells the ER appeared occasionally expanded in the vicinity of LDs (Fig 3A). In Fig 2 F panels I and II (wt cells) appear different from panel III (SKO). Are these representative images? Are the ER morphological alterations restricted to the ER-LD contacts or other morphological changes are seen in seipin KO cells?

**The ER in the vicinity of the LD contacts indeed appears sometimes expanded as shown in Fig. 3A. In addition, we assessed the overall ER diameter from WT and SKO cells after 1 h of OA loading. Stereological analysis showed that the ER also outside the LD contacts was significantly wider in SKO compared to WT cells (Fig 3C, D), suggesting a larger perturbation of ER morphology. We speculated that this may be related to abnormal deposition of neutral lipids within the ER bilayer but this clearly needs to be better addressed in future studies. The panels in Fig. 2F are actually not representative when it comes to the overall ER morphology. For Panel III (SKO), we chose an ER area where the reticulum was less dense, just to make the ER-independent movement of the LD highlighted in the exemplary video better visible.**

5- The STORM measurements found differences in the diameter of WT and A212P GFP structures. Did the differences reflect the association/contact with LDs or only structures in contact with LD were selected for the measurements?

**For the STORM measurements we imaged any WT- and A212P-seipin-GFP structures (these are single channel recordings, so we cannot identify whether the structures are associated with LDs or not). We have attempted to look at this by two-channel recordings. However, since the lipids within the LD might cause chromatic aberrations we are not ready to make definite conclusions. Regarding A212P vs WT-seipin, however, both immuno-EM quantification (Fig. 1G, 1H) and additional quantifications carried out from fluorescence micrographs (Fig. S10C) suggest that the A212P-seipin structures are less well concentrated at ER-LD contact sites compared to the wild-type protein.**

6- The authors detect a defect in neutral lipid synthesis during later stages of LD formation, which according to the literature should depend on DGAT2. Did the authors checked whether DGAT2 localizes in the KO cells?

**We have now added experimental data on DGAT2 localization in WT and seipin KO cells. We overexpressed a nontagged DGAT2 construct which we detected with a specific antibody, as endogenous DGAT2 was not detectable (we tested two antibodies). We found that DGAT2 localized only to a minor fraction of LDs at the early time (1 h) of OA loading, as described previously, in both WT and SKO cells. At the later time point (20 h of OA loading), the majority of large LDs in both WT and SKO cells recruited DGAT2, without obvious differences in DGAT2 recruitment between the genotypes (Fig S10H). This is analogous to earlier findings in seipin deficient yeast (Jacquier et al. J. Cell. Sci. 2011). Please note that we are not able to make very strong conclusions about the localization of DGAT2 since DGAT2 overexpression itself tends to increase the size of LDs in both WT and SKO cells (although it did not rescue the tiny LDs of SKO cells).**

7- The conclusions about the differences in localization of wt and A212P GFP-Seipin would be strengthened by showing that they are not simply due to different levels of expression. Also, how does the levels of these GFP fusion compare to endogenous Seipin?

**These are good points and we have now added more information related to the expression levels. The A212P-seipin-GFP mutant protein is expressed at roughly similar or slightly lower levels than the WT-seipin-GFP in the SKO background (Fig. S1G). This is reassuring considering that high overexpression is more typically associated with mislocalization. The level of seipin-GFP expression in these rescue cell lines is about 4-fold higher at the mRNA level than the endogenous seipin (Fig. S1F). We have now also added data on cells in which endogenous seipin is tagged with GFP at the genomic locus. These data (Figs 1I, Fig S4A-C, Fig 6A, Fig S10A-B) agree well with those obtained with the WT-seipin-GFP rescue cell line.**

8- In Supplementary Fig 5A, is it expected that delipidated cells still accumulate such high amounts of neutral lipids?

**In Fig. S5A, we are detecting Bodipy that has been fed to cells for 24 h after a 48 h delipidation. The upper bands labelled NL are identified based on the position of unlabeled triglycerides and cholesterol esters in the TLC system. It is conceivable that the Bodipy moiety drives Bodipy-C12 to preferentially incorporate into neutral lipids and thus the result may not reflect the amounts of unlabeled neutral lipids in these conditions.**

**Referee #2:**

This paper probes the functional role of Seipin at ER-LD contact sites in animal cells. Seipin is an integral ER membrane protein. Nice imaging data shown here localize Seipin to dynamic ER puncta. Nearly all LDs track together with a Seipin punctae at a MCS. They generate knockout Seipin cells (SKO) to test the effect of depletion on LD maturation, ER-LD tethering, and trafficking of lipids and proteins between ER-LD MCSs. SKO cells have smaller more abundant LDs, variable tethering, and can traffic lipids and proteins between the ER and small but not larger LDs. Suggests that LD size in SKO cells could be blocked because components stop trafficking from ER to LDs. Nice control, cytoplasmic proteins can still traffic to LD in SKO.

This story does a good job characterizing the phenotype of Seipin in animal cells. Not a new protein, Seipin has been linked to ER-LD contact functions before in yeast, where KO gives the opposite phenotype. Data shown here does a convincing job to demonstrate that Seipin KOs have reduced protein/lipid trafficking at ER-LDs. However some concerns must be addressed:

**We thank the Reviewer for the very positive and constructive remarks and address the remaining concerns below.**

1) One big issue is that I don't agree with the general conclusion that Seipin is a tether because on average SKO cells actually have increased ER-LD contact (just a minor population has lost contact). That is not the phenotype of a tether.

**The Reviewer is right. We have no direct evidence that seipin functions as a tether and indeed, the increased ER-LD contacts of some LDs in SKO cells do not really match with this idea. We have reformulated the manuscript text (p. 17, 19; highlighted with yellow) and have also changed the title of the manuscript accordingly.**

2) The quality of the EM data in F1G and Fig 4G is not sufficient for quantification. Membrane bound organelles, like LDs, should have smooth not angular lines, membranes appear smashed against each other by fixation, and how are they identifying the opposing membrane as ER when it is too wide to be ER and not ribosome bound?

**In the immuno-EM figures (Fig. 1G and 4G), the LD contours are unfortunately misshapen, as**

the reviewer points out. This is a technical caveat related to the compromise between preservation of morphological ultrastructure and antibody epitope accessibility. We employed mild chemical fixation to preserve the antigenicity of GFP under these conditions, and this inevitably resulted in partial deformation of the contours of membrane bound organelles. We have also tested immunolabeling of thin sections from high pressure frozen, freeze substituted cells – which preserves the organelle architecture better. Unfortunately, the antibodies no longer work under these conditions. Therefore, we cannot replace the immuno-EM data with other images. However, we have provided images with larger fields of view as supplements, which should help in the orientation and organelle (ER) identification (Fig S3). Please also note that even if the membrane preservation is not optimal, we have here always compared cells prepared identically. Thus, the same compromises resulting from the non-optimal preservation of membranous structures holds for all conditions.

3) LDs in SKOs look like background staining in F1A.

The vast majority of newly formed LDs in SKO cells tend to be somehow arrested in their growth beyond a diameter of about 200 nm, as our EM data indicate. Thus, at the resolution of light microscopy (Fig. 1A) the LDs are barely detectable and pending on the fluorescent tracer used, may escape detection. For instance, BODIPY hardly detects them and Nile red/oil red O staining certainly does not. So far, in our hands LD540 has been the most sensitive tracer to pick up the small nascent LDs of SKO cells, although we admit that the signal-noise ratio is not very high.

**Referee #3:**

In this manuscript, the authors characterize the role of WT Seipin in lipid droplet (LD) biogenesis and investigate how the absence of Seipin (SKO) affects LD biology in A431 cells. They aim to link it to a human pathological mutation in patients harboring a mutation in the Seipin gene (A212P) and they also use patient fibroblasts.

In summary, in the absence of Seipin, newly formed LDs are smaller, less associated with the ER and move faster. Seipin localizes to the ER/LD contact site. In the absence of Seipin, ACSL3 is not recruited to forming LDs (whereas the recruitment of probe Hpo and the lipid Bodipy are not affected). Conversely, the absence of Seipin affects the recruitment of Hop and Bodipy in "older"/formed LDs.

The paper is data rich and represents a lot of hard work. The experiments are well executed. The ms is primarily microscopy based. In particular, the authors use a number of very fancy/new light and electron microscopy based imaging techniques (although not all of them always completely justified).

The key protocol is to delipidate A431 cells (WT or CRISPR Seipin KO, SKO) overnight and feed them with large amount of oleic acid to stimulate acute lipid droplet biogenesis.

**We thank the Reviewer for the careful reading of our manuscript and for the constructive and very helpful remarks. Below, we address the specific comments made.**

1) The results are interesting and new, although with the publication of the yeast paper on the Seipin homolog last year, I would insist to have a more open comparison, what is similar and which new concepts this study brings forward. I would insist on this.

**We have now included more comprehensive discussion and more open comparison of our data to the other papers recently reported. We agree that the paper from Carvalho's lab on the yeast seipin homolog is very important, as is the very recent findings from Tobias Walther's and Robert Farese's lab on the drosophila and mammalian seipin. We have now more thoroughly discussed these findings in relation to our data (please see the revised Discussion, text highlighted with yellow).**

2) The main issue I have with these data is that the authors aim to link their data to a pathological situation.

- The cells used for these studies is not relevant to fat metabolism. A431 cells are of epidermoid carcinoma origin. At least they could have performed some experiments in adipocytes.
- Their experimental setting informs an acute situation, not a long term steady state as it is the case in patients. The simple fact that SKO clones were made and are viable means that its role in these cells is not as critical as in patients who are quite sick.
- Patients harbor a mutated Seipin whereas their experimental system lacks Seipin altogether. It is actually possible that the mutated protein is more toxic than the absence of the WT protein.

Altogether, I have a hard time understanding how the presented data on acute LD biogenesis explain the phenotype of 0% fat in patients?

Do these fast non ER associated LDs fail to get neutral lipid after neogenesis?

Can Seipin have two roles, one in LD biogenesis and another on formed/older LDs to recruit lipids, perhaps by keeping the tethering to the ER?

Data suggest that it might be the case (as Hpo and BDY C12 are not recruited to formed/older LDs in the absence of Seipin).

Therefore an analysis on older/steady state LDs would be necessary.

**We appreciate the reviewers' point that reductionist cell models do not adequately recapitulate the real pathobiology in patients and we have now pointed this out in the Discussion (last paragraph, p. 19). This holds true not only for the cancer cell models employed here but also for instance for yeast as a model for human pathophysiology. Nevertheless, we believe that valuable insights may be obtained from such reductionist models especially when it comes to conserved, basic cell biological mechanisms.**

**To reach better understanding of the consequences of seipin deficiency in longer term, we have now added data from older LDs (20 h of OA loading, Fig 6, Fig S10D-H)) as well as steady state conditions from cells cultivated in normal growth medium (Fig S1D, Fig S5C-E), also including primary BSCL2 patient fibroblasts (Fig S12A). These data reveal that the defects observed during acute LD biogenesis are also preserved under more chronic conditions. However, we wish to point out that in this paper the main focus is on the earliest defects observed when seipin is not functional. As there is substantially more heterogeneity in the LD phenotypes at later time points – in agreement with other investigators – a thorough characterization of later LDs under various metabolic cues constitutes a study of its own.**

**Regarding the reviewer's question whether the fast non-ER associated LDs fail to acquire neutral lipid after neogenesis, this is indeed what we assume is happening. Based on our data on older**

**LDs it also seems that seipin plays a role not only during LD biogenesis but also in older LDs, as they also show defects (discussed on p. 19). This is in line with the findings from other groups that have focused their studies on LDs at steady state/older LDs.**

Specific comments

3) In SKO, newly synthesized lipid droplets (LD) are numerous but smaller and moving faster, less directionally than WT. Furthermore, a larger subset of LDs are not associated with ER and those are the ones that move fast.

In my view, it is essential to quantify all these facts.

**In addition to quantifications of LD movement and ER association (Fig 2C-K, Fig 6E-F, Fig S10E-G), we have now added quantifications of LD sizes and areas (Fig 1B, S1B, 2J, 6C, S10D).**

3.1: Smaller LD in SKO: How much smaller? Is there an increase/decrease in total LD volume in WT versus SKO?

Is their composition different in lipids? In proteins? At steady state?

**In light microscopic analysis, we have indicated LD areas, as is typically done. In EM analysis, we have provided LD volumes (please see previous answer).**

3.2: What is the LD pattern in cells that are not challenged by delipidation and OA feeding?? Is there a difference between WT and SKO? What is this?

**This is a good point. We have now included data on the LD pattern of cells grown in normal growth medium (Fig. S1D). There is a clear difference between WT and SKO cells: WT LDs are on average larger and more uniform in size. In SKO cells there are tiny LDs, which move faster than the LDs of WT cells. We have now added data on this (S5C, 5D, 5E). Please note that in this manuscript we have mostly focused on the early events of LD biogenesis as this is where the earliest defects in LD morphology are detectable.**

3.3: Mobility is assessed during 6 seconds. This is not very long and reflects the mobility of newly formed LDs. What is the mobility of older LDs? Is there a difference between WT and SKO?

**We have been focusing on LD mobility for short time periods because the small LDs of SKO cells and BSCL2 fibroblasts display highly increased mobility. To track these LDs we need high frame**

rates of imaging and imaging the same cell with these regimes for longer periods of time tends to be phototoxic. We have now also included data on the mobility of older LDs (after 20 h of OA loading, Fig. 6E-F, Fig S10 E-G). Also under these conditions, there is a difference between WT and SKO cells, with very mobile tiny LDs present in SKO cells also after 20 h OA loading. As discussed in our response to Reviewer 1 (point 3), the fraction of small peripheral LDs that move apparently independently of the ER (and may therefore be detached from the ER) is roughly similar (about 20%) at both 1 h and 20 h of OA loading. Thus, our data suggest that this fraction does not change significantly over time. This may be a reflection of continuous formation defective of LDs in seipin deficient cells. The detached LDs may not accumulate if they give rise to additional LD “subtypes” observed more abundantly in later LDs, i.e. aggregated LDs and supersized LDs, and/or if they become degraded over time. We also found that the clustered LDs and larger LDs of WT and SKO cells are relatively more immobile than the smaller single LDs. In any case, for the present work it is interesting that the novel phenotype found, LDs detached from the ER, is not a transient intermediate but also observed when LDs have been present in cells for longer periods of time.

3.4: How many WT LDs dissociate from ER after biogenesis. Is the SKO phenotype not only an accelerated kinetics of detachment? Is it possible that Seipin stabilizes the contact?

**In WT cells, all the LDs analyzed remained associated with the ER. Therefore, the SKO phenotype appears not to be simply accelerated kinetics of detachment. Yes, it is indeed possible – and perhaps likely - that seipin stabilizes the contact, and we have discussed this possibility.**

4) WT Seipin localizes to LD. Nearly each LD is decorated by one spot of Seipin. The localization of the mutant protein with respect to LD is not investigated but it appears more ER.

4.1: Also not investigated is whether this Seipin dot on LDs is stable over time? As mentioned above, the study looks at the acute formation of LDs but it would be important (with respect to the clinical situation) to show whether older LDs contain Seipin as well, how long Seipin remains to LDs, whether it changes, how, etc.

**As requested, we have now addressed the seipin localization on older LDs (20 h OA loading). We found that seipin also localizes to older LDs and appears to remain dynamically but stably associated with them, similarly as with nascent LDs (Fig 6A, S10A-B). The A212P-seipin mutant was less efficiently associated with LDs also after 20 h of OA loading (Fig S10C-D). Please note,**

however, that with the rapid dynamics of seipin, fast frame-rate recordings are necessary and phototoxicity and photobleaching limit the time that any single LD and associated seipin structure can be followed. We speculate (discussion, p. 19) that seipin may indeed play a role also in the maintenance of the ER-LD contacts of more mature LDs. However, since the defects in SKO cells are numerous already at the early steps of LD biogenesis, looking at older LDs in these cells might not provide a definite answer for this role. To fully address this, a tightly inducible KO system would be necessary.

4.2: Using STORM, The authors show that the structure decorated by WT Seipin is 15% larger than this decorated by mutant Seipin. This is an unnecessary piece of information and looks more like a technological display. Please place this in Suppl figure.

**We have now placed this information in the Supplement, as requested (Fig S2B).**

4.3: Last, by EM, WT Seipin is confirmed to localize at LD/ER contact whereas mutant Seipin appears restricted to the ER as suggested by the IF analysis.

Same comment as above regarding older LDs.

**As discussed above (response to point 4.1), we have now addressed this by imaging the SKO + WT or A212P-seipin cells. Also older LDs harbor WT-seipin, but many older LDs do not have A212P-seipin at their ER-LD contacts (Fig S10C).**

5) The LD/ER contacts are then investigated in more details using 2 different EM methods.

The first show that in a WT situation, LDs contact the ER at discrete site whereas in SKO, more and smaller LDs are away from ER, thus confirming the light microscopy data.

The second shows that in WT, the LD/ER contacts are refined whereas in SKO, when LDs are in contact with ER, the contact area is larger. Those LD appear wrapped up. Thus the authors reach the same conclusion as in yeast (Grippa et al 2015).

However, they also found examples of flimsy contacts, perhaps from LDs that are not tethered properly and leave the ER vicinity.

**The similarity of ER-LD enwrappings in our data and in yeast is a good point and we have cited Grippa et al., 2015 in this context (p. 18). It is indeed attractive to think that the flimsy contacts may represent those LDs that are not properly tethered and leave the ER vicinity. In order to**

**improve the quantitiveness of our data, we have included quantification of the fraction of LDs harboring flimsy contacts (p. 9).**

6) What is the relevance of the defects in LD/ER contacts?

The authors investigate the recruitment of ACSL3 to LDs, a protein that are reported to mark lipogenic ER subdomains and nascent LDs.

- In the absence of Seipin ACSL3 is not recruited to LDs that are here mark by protein ADRP (tagged).

-Using an elegant heterogous cell fusion, this recruitment is shown to be generated during LD biogenesis. Seipin needs to be present during LD formation to allow ACSL3 recruitment to LDs.

-Endogenous ACSL3 behaves the same as tagged ACSL3. EM reveals that ACSL3 decorates the entire surface of LDs in WT cells whereas it remains at contact sites in SKO and does not appear to diffuse in the plane of the LD monolayer.

This figure (Fig.4) is very oddly presented. I suggest to present data on the endogenous ACSL3 first moving to the tagged version data (A, B) to the suppl, especially because the introduction of yet another marker ADPR makes the ms more complicated and to my view, does not add much. The cell fusion (C) is interesting and informative but after the endogenous data.

**We considered moving the data on tagged proteins to the Supplement but thought that it might be confusing to present the cell fusion data without first showing the localization of the overexpressed markers in the respective individual cells (before fusion). Furthermore, ADRP serves as a marker of a protein that is recruited to LDs from the cytoplasm. Therefore, we ended up keeping the figure as it was.**

7) The authors then re-investigate the ER to LD trafficking via Seipin using a specific probe called Hpo-Cherry and the lipid BDY-C12

In their cases, it is the opposite to ACSL3. Seipin is not required for the targeting of these (artificial) reagents to LDs during the biogenesis of LDs, but Seipin is required for them to reach older/formed LDs. (as a reminder, for ACSL3, Seipin is required its targeting to LDs during their biogenesis).

This is hard to reconcile with the model proposed by the authors at the end of Figure 6 focusing on ACSL3 in term of LD maturation and acquisition of lipids.

The data on the probes/lipids suggest that Seipin has a second role on older LDs and I would argue that

that this role is probably more relevant for the disease.

Again, are those older LDs still associated with the ER? What is the difference between WT and SKO older LDs?

**Thank you for these considerations. We have included more information to our model to hopefully make it more understandable (Fig 8). However, we have there focused on the early events in LD biogenesis, as this is what most our findings are related to. Please note that we have now also included characterization of LDs at a later time point (20h), including seipin LD association, ACSL3 and DGAT2 recruitment and model cargo protein (HPos) FRAP recovery (Fig 6A-J and Fig S10A-H). These findings suggest that the LD phenotypes become more heterogeneous over time. While ACSL3 and DGAT2 can reach the large LDs that are more prevalent at later time points in SKO cells, there are also small LDs not properly connected to the ER as well as “giant” LDs, some of which may have strong ER connectivity based on HPos recovery. However, as discussed above, at this stage we are not ready to make conclusions about the role of seipin in older LDs due to the difficulties in distinguishing between primary and secondary defects in its absence.**

8) The title should mentioned newly formed LDs and the abstract should explain that ACSL3 and Hpo and BDY-C12 behaves differently.

**We have changed the title as requested. Due to the length limit of the abstract we could unfortunately not incorporate the names of the individual markers studied but we hope that the more explicit legend of the summary figure is helpful.**

Thank you for submitting the revised version of your manuscript to us. It has now been seen again by three referees whose comments are enclosed. As you will see, all three referees express interest in your manuscript and are broadly in favour of publication, pending satisfactory minor revision.

I would thus like to invite you to address the remaining concerns by providing a point-by-point response. Note that it is fine to include your STORM data in the manuscript (referee #1, second paragraph). Please address points 2 and 3 mentioned by referee #2 - I'd suggest to clearly state in the manuscript text that the immuno-EM data have to be interpreted with caution and might not be definitive. Please also discuss the LD size-contact site size % issue in the manuscript text.

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## REFeree REPORTS

### Referee #1:

My major concern with the initial version of this paper was related with the function of Seipin tethering lipid droplets to the ER. I believe that the interpretation presented in this version is more adequate. Thus I have no other major concerns.

There is a minor, peripheral issue that the authors may want to consider. This is related with the STORM analysis of wt and mutant A212 mutant. Since it is unclear whether the changes in size reflect differences in localization, proximity to LDs, oligomerization state or other, in my mind the data is not informative. Perhaps the authors should consider to remove it from the manuscript.

### Referee #2:

1. In the revised version of the manuscript the authors addressed my concern that seipin is not acting as a tether by removing this statement. I am glad that they have done so since the data do not support this conclusions, but the findings seem thus not to agree with the yeast model. The novelty of the findings presented in the revised version is not high.
2. I am still concerned by the quantifications performed on the immuno-EM, it is not acceptable to argue that the quality of the EM is equally bad between control and seipin sko.
3. The seipin sko results in smaller LDs, and yet the authors compare % surface area covered by ER for LDs between wt and seipin sko. A larger % surface area covered on a smaller LD might have the same total ER-LD surface area. Its like holding a small ball vs. a big ball with your hand. Sure your hand contacts more of the small ball but your hand is the same size. So it may be that the seipin Sko does not affect contact site size.

### Referee #3:

The authors have done a good job in answering my comments and clarify a number of points including the study of the large LDs in SKO. They also changed the title to make it more precise.

As a result, I find that the manuscript is suitable for publication to the EMBO J.

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There is a minor, peripheral issue that the authors may want to consider. This is related with the STORM analysis of wt and mutant A212 mutant. Since it is unclear whether the changes in size reflect differences in localization, proximity to LDs, oligomerization state or other, in my mind the data is not informative. Perhaps the authors should consider to remove it from the manuscript.

**Thank you for the favorable remarks on our revised manuscript. We appreciate the limitations of the STORM data as pointed out by the reviewer, but thought that it might nevertheless be interesting, since other investigators have studied the size of seipin (WT vs. A212P mutant) oligomers in vitro using purified proteins, and our findings from cells fit with these results. We have therefore kept this data in the manuscript.**

Referee #2:

1. In the revised version of the manuscript the authors addressed my concern that seipin is not acting as a tether by removing this statement. I am glad that they have done so since the data do not support this conclusion, but the findings seem thus not to agree with the yeast model. The novelty of the findings presented in the revised version is not high.

**We thank the reviewer for helping us revise the interpretation of our data.**

2. I am still concerned by the quantifications performed on the immuno-EM, it is not acceptable to argue that the quality of the EM is equally bad between control and seipin sko.

**We understand that the immuno-EM data might not be definitive because of the technical limitations involved, and have now added in the manuscript text a statement that these data should be interpreted with caution (page 6).**

3. The seipin sko results in smaller LDs, and yet the authors compare % surface area covered by ER for LDs between wt and seipin sko. A larger % surface area covered on a smaller LD might have the same total ER-LD surface area. It's like holding a small ball vs. a big ball with your hand. Sure your hand contacts more of the small ball but your hand is the same size. So it may be that the seipin Sko does not affect contact site size.

**This is a very good point. Thank you for this comment. We have now included the data on the sizes of the contacts (not just their fraction of the total LD surfaces). These results show that there is a substantially larger heterogeneity in the actual surface area of ER-LD contacts in seipin KO cells (page 8).**

Referee #3:

The authors have done a good job in answering my comments and clarify a number of points including the study of the large LDs in SKO.

They also changed the title to make it more precise.

As a result, I find that the manuscript is suitable for publication to the EMBO J.

**Thank you for helping us improve the manuscript.**

3rd Editorial Decision

24 October 2016

Thank you for submitting the final version of your manuscript to us. I appreciate the introduced changes, and I am happy to accept your manuscript for publication in The EMBO Journal.

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Elina Ikonen

Journal Submitted to: The EMBO Journal

Manuscript Number: EMBOJ-2016-95170

**Reporting Checklist For Life Sciences Articles (Rev. July 2015)**

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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

**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?                                                                                     | Not applicable                                                                                                                                                                                                                                                                                                                                                                    |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.                                                                       | Not applicable                                                                                                                                                                                                                                                                                                                                                                    |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?                                                      | For some of the assays we employed pre-established inclusion/exclusion criteria. For example, we excluded high expressing cells from analysis to confer specificity (Page 6 of manuscript text, see also Appendix Figure S5 legend). For imaging of ER-LD associated movement, we focused on peripheral areas of ER to be able to track individual ER and LD structures (page 7). |
| 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.                | Not applicable                                                                                                                                                                                                                                                                                                                                                                    |
| For animal studies, include a statement about randomization even if no randomization was used.                                                                                          | Not applicable                                                                                                                                                                                                                                                                                                                                                                    |
| 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. | Yes. Stereological analysis was used whenever possible for EM (see appendix materials and methods). Manual analysis of cluster size in STORM was performed in a blinded fashion. Also a substantial amount of the LD tracking and ACSL3 staining was done done by a blinded investigator who was not aware of the sample identities.                                              |
| 4.b. For animal studies, include a statement about blinding even if no blinding was done                                                                                                | Not applicable                                                                                                                                                                                                                                                                                                                                                                    |
| 5. For every figure, are statistical tests justified as appropriate?                                                                                                                    | To the best of our understanding yes.                                                                                                                                                                                                                                                                                                                                             |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.                                                                      | Normality of data was assessed with D'Agostino & Pearson omnibus normality test and parametric or non-parametric tests indicated were used accordingly.                                                                                                                                                                                                                           |
| Is there an estimate of variation within each group of data?                                                                                                                            | F-test was used to assess for variance and t-tests assuming or not assuming equal variance were used accordingly.                                                                                                                                                                                                                                                                 |
| Is the variance similar between the groups that are being statistically compared?                                                                                                       | Not always, and thus t-tests not assuming equal variance were employed.                                                                                                                                                                                                                                                                                                           |

**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>  
  
<http://grants.nih.gov/grants/olaw/olaw.htm>  
<http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm>  
<http://ClinicalTrials.gov>  
<http://www.consort-statement.org>  
<http://www.consort-statement.org/checklists/view/32-consort/66-title>  
  
<http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun>  
  
<http://datadryad.org>  
  
<http://figshare.com>  
  
<http://www.ncbi.nlm.nih.gov/gap>  
  
<http://www.ebi.ac.uk/ega>  
  
<http://biomodels.net/>  
  
<http://biomodels.net/miriam/>  
<http://jii.biochem.sun.ac.za>  
[http://oba.od.nih.gov/biosecurity/biosecurity\\_documents.html](http://oba.od.nih.gov/biosecurity/biosecurity_documents.html)  
<http://www.selectagents.gov/>

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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). | Antibody sources/catalog numbers are given in the supplementary information (Appendix methods and materials page 2). ACSL3 antibody: <a href="https://www.antibodypedia.com/gene/1945/ACSL3/antibody/344523/H00002181-B01P">https://www.antibodypedia.com/gene/1945/ACSL3/antibody/344523/H00002181-B01P</a> Catalog number is in Materials and methods. For IF in A431 cells we also validated it with siRNAs (Fig S7B). Rabbit anti-GFP antibody: <a href="http://www.abcam.com/gfp-antibody-chip-grade-ab290.html">http://www.abcam.com/gfp-antibody-chip-grade-ab290.html</a> is very widely used (circa 900 publications cite this antibody) and we also validated it for our STORM experiments (Fig S2A) and Western blotting (Fig S4). anti-DGAT2 antibody has been described and validated (Krueshner et al 2008) and we cite that paper. Mouse anti-GFP profile can be found at 1degreebio: <a href="http://1degreebio.org/reagents/product/570956/?qid=1402371">http://1degreebio.org/reagents/product/570956/?qid=1402371</a> . anti-calreticulin antibody (PA3-900) is also widely used, with at least 150 citations in antibodypedia: <a href="https://www.antibodypedia.com/gene/1028/CALR/antibody/561365/PA3-900">https://www.antibodypedia.com/gene/1028/CALR/antibody/561365/PA3-900</a> |
| 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.                                                                                                                                                                        | A431 cells were from ATCC. Ctrl primary fibroblasts were from Coriell Cell Repository and patient fibroblasts from a collaborating group, with the relevant publication cited (see page 20 of manuscript text). All cell lines used in our lab are regularly tested for mycoplasma contamination with PCR.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |

\* 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.                                                                                                                                                                                                      | Not applicable |
| 9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.                                                                                                                                                                                                                 | Not applicable |
| 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. | Not applicable |

#### E- Human Subjects

|                                                                                                                                                                                                                                                                                                                    |                                                                             |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------|
| 11. Identify the committee(s) approving the study protocol.                                                                                                                                                                                                                                                        | Ethical permits obtained by co-authors. For details see Boutet et al. 2009. |
| 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.                                                            | See above.                                                                  |
| 13. For publication of patient photos, include a statement confirming that consent to publish was obtained.                                                                                                                                                                                                        | Not applicable                                                              |
| 14. Report any restrictions on the availability (and/or on the use) of human data or samples.                                                                                                                                                                                                                      | Upon permission from co-authors Corinne Vigouroux and Jocelyne Magré.       |
| 15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.                                                                                                                                                                                                         | Not applicable                                                              |
| 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. | Not applicable                                                              |
| 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.                                                                        | Not applicable                                                              |

#### F- Data Accessibility

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------|
| 18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions                                                                                                                                                                                                                                                                                                                                   | Not applicable                 |
| 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).                                                                                                                                                                                                                            | See supplementary information. |
| 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).                                                                                                                                                                                                                     | Not applicable                 |
| 21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section.<br><br>Examples:<br><b>Primary Data</b><br>Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in <i>Shewanella oneidensis</i> MR-1. Gene Expression Omnibus GSE39462<br><b>Referenced Data</b><br>Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CRA/5 of TR. Protein Data Bank 4O26<br>AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208                                            | Not applicable                 |
| 22. 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 Biocompare (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. | Not applicable                 |

#### G- Dual use research of concern

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|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| 23. 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. |
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