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Identification of MOSPD2, a novel scaffold for endoplasmic reticulum membrane contact sites

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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.)

1st Editorial Decision 12 December 2017

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

As you will see, the referees acknowledge the potential interest of the findings but they also raise a number of concerns and have a number of suggestions on how the study could be strengthened. Going through the comments, it appears that there are four core sets of experiments/concerns:

1) All referees agree that Figure 1A does not provide sufficient evidence that contact sites are still present in VAP-depleted cells.
Upon further discussion with the referees, we suggest to remove Figure 1A and to refer to published work instead that provided evidence for VAPA/B-independent tethers.

2) The manuscript does not provide evidence that MOSPD2 is required for contact site formation.
3) What is the relative contribution of MOSPD2 and VAP-A/B to contact site formation?

Again, upon further discussion with the referees, we suggest to provide a quantification of contact sites in single and/or double/triple mutants to provide further information on the role of MOSPD2 as tether in comparison to VAPA/B. Also, if the number of contact sites is not altered in MOSPD2-depleted cells this could be reported.

4) What is the main interaction partner for MOSPD2 in cells and does MOSPD2 compete with VAP-A/B for binding to FFAT motif-containing proteins?

We have also discussed this point further and while this information will certainly be of interest and could be included, it is not essential to address these points experimentally. These experiments might be the focus of a further study.

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

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

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Regarding data quantification, please ensure to specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in all respective figure legends. Please also include scale bars in all microscopy images.

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

When submitting your revised manuscript, we will require:

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

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

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

REFEREE REPORTS

Referee #1:

The manuscript by Mattia et al reports on identification of a novel tethering molecule on the ER membrane that is similar to the well-known and highly studied VAPA/B. They show that the new tether, MOSPD2 has an MSP domain (similarly to VAPs) and, using beautiful homology modeling and recombinant protein binding assays also show that it binds FFAT motifs similarly to VAPS. Moreover, they demonstrate that MOSPD2 resides in the ER membrane (thanks to a C' TMD) and has the capacity to bind, directly, FFAT (or FFAT like) motif containing proteins on opposing membranes such as mitochondria, endosomes and Golgi. Moreover, they elegantly show that ectopic expression of such proteins recruits MOSPD2 to contact sites and strengthens the association between the organelle membranes. Thus this paper convincingly demonstrates that MOSPD2 is a parallel pathway to VAPs in creating ER centric contact sites.

The field of contact sites is now erupting with discoveries on new tethering molecules. This new aspect of cell biology is fascinating and highly relevant and this manuscript is a beautiful demonstration on how such tethers can be identified and verified. It is performed at a very high standard and will be an extremely important additioni to the field, hence I strongly support its publication in EMBO reports following a few, very minor, changes and suggestions:

1. Figure 1A - with no quantification this figure does not support the notion that silencing of VAPA/B does not lead to loss of contact sites. To make this point the silenced lines must be compared to a control and quantification should be present. However, I feel like this point is not important and the whole panel can be removed from the paper without, in anyway, affecting the main message. Hence to clarify - I am not asking for another experiment but I do think this panel should be either modified or removed.
2. It is very confusing that in the figures panels are labeled as uppercase letters (A) and then subpanels are labeled in lower case (a) as when reading the legends one often reads the wrong legend. I suggest to either use roman numerals for sub-panels or make each sub-panel be an independent panel and have its own uppercase letter or just not number them.
3. Figure 3C shows a model of MOSPD2 and the position of the RD/LD mutant but the mutant is already used in Figure 2 hence I think it is best to put this model in the previous figure when the mutant is first presented.
4. The authors perform beautiful assays to show which proteins MOSPD2 CAN interact with and by which domains. While these are all very well performed, they do not report on what the main interactor of MOSPD2 is in its natural context or what does MOSPD2 ACTUALLY bind. I would strongly recommend that the authors pull down MOSPD2 and the RD/LD mutant and show which protein is the main binding partner of MOSPD2 in normal cells. Having said that - this is not essential to the main point of the paper, which is very well supported and hence if this is a lengthy and complicated experiment I would just ask that the authors discuss the fact that the actual interacting partners of MOSPD2 in the native context have not yet been identified.

Referee #2:

Summary
The submitted manuscript proposes that MOSPD2 is a novel tether connecting the ER with various organelles. Using elegant approaches, Di Mattia et al. clearly demonstrate that MOSPD2 is an ER-resident protein that binds FFAT-containing proteins. This is a novel and exciting advance within the field. However, the authors have not provided any evidence that MOSPD2 is necessary for formation of contact sites between the ER and other organelles within cells. As it stands, it is difficult to conclude that MOSPD2 is a bona fide tether.

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Major comments
1. The authors start their narrative by documenting contact sites between ER and mitochondria/endosomes in VAP-depleted cells (Fig. 1A) and use this result to suggest the presence of VAP-independent tethers. What is the extent of knockdown? Is this documented in Fig. S4? Where is the control(!)? And where is the quantification? This basic characterisation is needed. Indeed, to truly establish whether MOSPD2 is involved in the formation of membrane contact sites the authors should quantitatively analyse contact site formation by electron microscopy after MOSPD2 knockdown/silencing as they have began to do with VAPs. Although technically challenging, localising MOSPD2 to contact sites with electron microscopy would also be nice.
2. In figures 1, 2, 3, 4, 5 and 7 the number of experimental repeats have not been made clear, these need to be added to the figure legends.
Minor comments
1. Page 3. Please cite appropriate reference(s) for contact sites and Ca2+ at end of para.
2. Fig. 1D. Presumably MOSPD2 was identified from the 60 kDa band. Please make this clear. Was this the highest scoring hit (same question for VAPA/B from the 30kDa band) ie were these proteins identified in an unbiased way or were the authors more selective.
3. Figure 2Aa.The colours in the figure, don't match the colours described in the legend (MOSPD2 is green in the figure, but the legend states it's orange).
4. Figure 2Ca. Please comment on the size shifts for the mutants.
5. Figure S1A. Does MOSPD2 have any obvious ER retention signals? Did the authors test whether the deltaC construct can also be relolcated upon coexpression with FFAT-proteins? This would be nice to know.
6. Ironically, the interaction between MOSPD2 and ORP1L is the least convincing at the IP (Figure 4C) and cellular (Fig. S3) level given that the FFAT motif used for identification is from an ORP!
7. Figure 7. The immunostaining of endogenous MOSPD2 is not overly convincing as the residual staining is significant and worrying ER-like in the example shown. Did the authors test whether relocation of endogenous MOSPD2 is FFAT dependent? Please further quantify colocalization with correlation coefficients. Does the endogenous protein coIP with FFAT-proteins?
8. Figure S2 legend has been duplicated.
9. LAMP1 is a late-endosome/lysosome marker.
10. Please add molecular mass markers to all blots.

Referee #3:

In this manuscript Di Mattia and colleagues reported the identification of a VAP-related protein, MOSPD2, that possibly plays a role in tethering between the ER and other organelles. Although the data presented are solid and prove that MOSPD2 binds FFAT motifs and can link lipid membranes in vitro, the relationship between MOSPD2 and VAPA/B in the context of the formation of membrane contact sites (MCS) has not been fully investigated.

1. To what extent MOSPD2 contributes to the formation of MCS between the ER and other organelles? Do MOSPD2 and VAPA/B independently, additively, or synergistically mediate membrane tethering? Are MCS disrupted upon depletion of only MOSPD2? What about VAPA/B and MOSPD2 double knockdown?

2. How about the specificity of target organelles? Are organelles linked by MOSPD2 the same as those by VAPA/B?

3.Does MOSPD2 compete with VAPA/B for binding to FFAT motif-containing proteins?

4.Is MOSPD2 co-localized with VAPA/B at MCS?

5.Regarding Fig. 1A: If the authors used this data as evidence that ER-mitochondria contacts are still present in VAPA/B-depleted cells, the data should be quantified.

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ANSWER TO POINT 1:
As suggested, we removed this panel from Figure 1. The literature suggesting the existence of VAP-A/VAP-B independent tethers is now referred in the first paragraph of the Results section: "Consistent with the notion that VAP-independent tethering mechanisms exist, VAP silencing only has a moderate effect on contacts involving the ER. For instance, loss of VAP-B only reduces ER-mitochondria contacts by 30% (Stoica et al., 2014), and the combined loss of VAP-A and VAP-B only decreases by half contacts with the ER in a sub-population of endosomes and in lysosomes (Eden et al., 2016)."
In addition, we conducted the experiment (now in Figure 9), but since our results are consistent with the cited literature, we preferred not mentioning it at that point in the manuscript.

2) The manuscript does not provide evidence that MOSPD2 is required for contact site formation.

ANSWER TO POINT 2:
We think that we provide several evidence that MOSPD2 is required for contact site formation:

- To the issue: is MOSPD2 mandatory for contact site formation?

We have used quantitative electron microscopy and showed that silencing MOSPD2 affects ER-endosome contacts (Fig. 9, Fig EV8). The effect we observed was stronger than the one seen upon VAP-A and VAP-B silencing (Fig. 9).
Still, MOSPD2 silencing does not completely erase membrane contact sites between the ER and organelles like mitochondria and endosomes (Fig. 9, Fig EV8). In fact, cells lacking MOSPD2 maintain a similar number of contact sites between the ER and the mitochondria (Fig. 9G). This finding is consistent with the results we obtained by silencing VAP proteins. At steady state, in HeLa cells, the loss of VAP proteins does not alter significantly membrane contact sites involving the ER and mitochondria (Fig. 9G). In this study, neither MOSPD2 nor VAP are absolutely required for the formation of membrane contact sites. Several possibilities can explain this result, notably one can evoke feedback control and/or redundancy. It is now clear that a number of discrete tether can build membrane contact sites between the ER and a variety of organelles. This was noted by reviewer 1 *"The field of contact sites is now erupting with discoveries on new tethering molecules"*. For instance Mitofusin 1 and 2 build contacts between the ER and mitochondria (Brito and Scorrano, 2008); Protrudin and Rab7 between ER and endosomes (Raiborg et al., 2015). It is then likely that membrane contact sites formation involves several tethers, and the loss of one tether is compensated. This idea was for instance nicely demonstrated in yeast (Manford et al, Dev Cell, 2012), in which the loss of 6 distinct proteins is required to decrease ER-plasma membrane contacts by 95%. Along the same idea, the contribution of specific tethers like MOSPD2 or VAP to membrane contact sites is probably cell-type and context dependent. For instance, quantification of VAP and MOSPD2 protein levels in HeLa cells, show that VAP-A and VAP-B proteins are more abundant than MOSPD2 by 200 and 7 fold (Fig. EV2). This observation supports the notion that membrane contact sites are diverse, probably dynamic/plastic, and they may evolve in response to a variety of molecular mechanisms.

- To the issue, is MOSPD2 able to build contact sites?

I think that we have produced many evidence of this in the manuscript. In brief, in vivo we showed that MOSPD2 mediates the recruitment of several organelles to the ER via a molecular mechanism that depends on the interaction between the MSP domain and the FFAT motif (Fig. 6, Fig. 7, Fig. 8). Moreover, using a defined in vitro approach, where liposomes bearing the MSP domain of MOSPD2 are mixed with target liposomes exposing a FFAT motif at their surface, MOSPD2 is directly recruiting target liposomes in the absence of any other protein (Fig. 3). This experiment shows that MOSPD2 is a genuine tether. Finally, in a complete new set of experiments we show that silencing of MOSPD2 specifically reduces (Fig. 9) ER-endosomes contacts (see below).

3) What is the relative contribution of MOSPD2 and VAP-A/B to contact site formation?
Again, upon further discussion with the referees, we suggest to provide a quantification of contact sites in single and/or double/triple mutants to provide further information on the role of MOSPD2 as tether in comparison to VAPA/B. Also, if the number of contact sites is not altered in MOSPD2-depleted cells this could be reported.

ANSWER TO POINT 3:

We have experimentally studied the contribution of MOSPD2 and VAP to the formation of ER-mitochondria and ER-endosome contacts by electron microscopy and stereology in cells silenced for VAP, MOSPD2, and VAP plus MOSPD2. The results are presented in the figure 9 of the manuscript and are commented in the result section and in the discussion. In brief, silencing them individually or together does not modify ER-mitochondria contacts, measured by stereology. However, ER-endosome contacts were affected by MOSPD2 silencing. Moreover, it revealed an additional function for MOSPD2 as its silencing specifically increased endosome-endosome contacts. Further studies will be necessary to understand this phenomenon.

4) What is the main interaction partner for MOSPD2 in cells and does MOSPD2 compete with VAP-A/B for binding to FFAT motif-containing proteins?
We have also discussed this point further and while this information will certainly be of interest and could be included, it is not essential to address these points experimentally. These experiments might be the focus of a further study.

ANSWER TO POINT 4:
To answer this question, we performed three sets of complementary experiments.
First, to identify the preferred MOSPD2 protein partner, we used an unbiased approach; we performed MOSPD2 immunoprecipitation and identified the interaction partners by mass spectrometry. A list of the 5 proteins containing a FFAT motif and identified by MS based on their ability to interact with WT MOSPD2, but not with the RD/LD MOSPD2 mutant, is now shown Fig 5A. In addition, a list of 109 proteins identified in this experiment is included in the source data of this manuscript.
Second, to directly study the competition between VAPs and MOSPD2 for the FFAT motif, we used an *in vitro* approach and measured their affinity for the canonical FFAT motif of ORP1 by surface plasmon resonance. Experiments are now presented in Fig. 3. In brief, the MSP domain of MOSPD2 has an affinity that is comparable to that of the MSP domain of VAP-A and VAP-B. This is consistent with the structural similarities of the MSP fold in both proteins (Fig. 2). All these results are presented in Fig. 3 panels A to H, Fig 5A and commented in the result section and in the discussion.
Third, we co-labeled endogenous VAP-A and endogenous MOSPD2 in cells expressing STARD3NL or the FFAT-defective mutant STARD3NL DFFAT. This data are now shown Fig. EV7. Interestingly, endogenous VAP-A was concentrated together with endogenous MOSPD2 in sub regions of the ER localized around STARD3NL positive endosomes, in a FFAT-dependent manner. Thus, MOSPD2 and VAP are present in the same contact sites.
Altogether, these experiments suggest that MOSPD2 and VAP compete for the interaction with FFAT-containing proteins.

Referee #1:

The manuscript by Mattia et al reports on identification of a novel tethering molecule on the ER membrane that is similar to the well-known and highly studied VAPA/B. They show that the new tether, MOSPD2 has an MSP domain (similarly to VAPs) and, using beautiful homology modeling and recombinant protein binding assays also show that it binds FFAT motifs similarly to VAPS. Moreover, they demonstrate that MOSPD2 resides in the ER membrane (thanks to a C' TMD) and has the capacity to bind, directly, FFAT (or FFAT like) motif containing proteins on opposing membranes such as mitochondria, endosomes and Golgi. Moreover, they elegantly show that ectopic expression of such proteins recruits MOSPD2 to contact sites and strengthens the association between the organelle membranes. Thus this paper convincingly demonstrates that MOSPD2 is a parallel pathway to VAPs in creating ER centric contact sites.

The field of contact sites is now erupting with discoveries on new tethering molecules. This new aspect of cell biology is fascinating and highly relevant and this manuscript is a beautiful demonstration on how such tethers can be identified and verified. It is performed at a very high standard and will be an extremely important additioni to the field, hence I strongly support its publication in EMBO reports following a few, very minor, changes and suggestions:

1. Figure 1A - with no quantification this figure does not support the notion that silencing of VAPA/B does not lead to loss of contact sites. To make this point the silenced lines must be

compared to a control and quantification should be present. However, I feel like this point is not important and the whole panel can be removed from the paper without, in anyway, affecting the main message. Hence to clarify - I am not asking for another experiment but I do think this panel should be either modified or removed.

This point is discussed in ANSWER TO POINT 1. As suggested, the panel was removed.

2. It is very confusing that in the figures panels are labeled as uppercase letters (A) and then subpanels are labeled in lower case (a) as when reading the legends one often reads the wrong legend. I suggest to either use roman numerals for sub-panels or make each sub-panel be an independent panel and have its own uppercase letter or just not number them.

The labeling of the figure panels was modified according to the reviewer's recommendations. Lowercase letter labels were either removed or replaced by uppercase letters.

3. Figure 3C shows a model of MOSPD2 and the position of the RD/LD mutant but the mutant is already used in Figure 2 hence I think it is best to put this model in the previous figure when the mutant is first presented.

Accordingly, the model of MOSPD2 and the position of the RD/LD mutant was moved to Figure 2.

4. The authors perform beautiful assays to show which proteins MOSPD2 CAN interact with and by which domains. While these are all very well performed, they do not report on what the main interactor of MOSPD2 is in its natural context or what does MOSPD2 ACTUALLY bind. I would strongly recommend that the authors pull down MOSPD2 and the RD/LD mutant and show which protein is the main binding partner of MOSPD2 in normal cells. Having said that - this is not essential to the main point of the paper, which is very well supported and hence if this is a lengthy and complicated experiment I would just ask that the authors discuss the fact that the actual interacting partners of MOSPD2 in the native context have not yet been identified.

This point is addressed above in the response to the editor (ANSWER TO POINT 4). As recommended, we performed pull down experiments using MOSPD2 and the RD/LD mutant.

Referee #2:

Summary
The submitted manuscript proposes that MOSPD2 is a novel tether connecting the ER with various organelles. Using elegant approaches, Di Mattia et al. clearly demonstrate that MOSPD2 is an ER-resident protein that binds FFAT-containing proteins. This is a novel and exciting advance within the field. However, the authors have not provided any evidence that MOSPD2 is necessary for formation of contact sites between the ER and other organelles within cells. As it stands, it is difficult to conclude that MOSPD2 is a bona fide tether.
Major comments
***1.** The authors start their narrative by documenting contact sites between ER and mitochondria/endosomes in VAP-depleted cells (Fig. 1A) and use this result to suggest the presence of VAP-independent tethers. What is the extent of knockdown? Is this documented in Fig. S4? Where is the control(!)? And where is the quantification? This basic characterisation is needed. Indeed, to truly establish whether MOSPD2 is involved in the formation of membrane contact sites the authors should quantitatively analyse contact site formation by electron microscopy after MOSPD2 knockdown/silencing as they have began to do with VAPs. Although technically challenging, localising MOSPD2 to contact sites with electron microscopy would also be nice.*

The first part of this comment is addressed in ANSWER TO THE EDITOR POINT 1. The panel shown Fig. 1A has been removed. Still, the cell lines knocked-down for VAP-A and VAP-B are present in Fig. EV5 (former Fig. S4). The extent of knock-down is shown in Fig. EV5B. The different Western blots used for this quantification are available as Source data for Fig. EV5.
The second part of this comment relates to the analysis of contact site formation in MOSPD2 knockdown cells. These experiments were performed (Now in Fig. 9 in the paper) and are commented in ANSWER TO POINT 3 above. We agree with the reviewer and it would be nice to

see MOSPD2 at contact sites but unfortunately we are not equipped to perform this kind of experiments.

2. In figures 1, 2, 3, 4, 5 and 7 the number of experimental repeats have not been made clear, these need to be added to the figure legends.
We have added the number of independent experiments performed in the figure legends. Figure 1E: Representative illustration of at least two independent experiments. Figure 2F: Representative illustration of at least two independent experiments. Figure 3J: Representative experiment of at least 3 independent experiments. Figure 4B: 20 cells from three independent experiments. Figure 7D: from three independent experiments. Figure 8B: n: three independent experiments. Figure 8G: from three independent experiments.

Minor comments
1. Page 3. Please cite appropriate reference(s) for contact sites and Ca2+ at end of para.

This paragraph concerns the involvement of VAP proteins in MCS having different roles, one of which is calcium transport. We have added the reference Paillusson et al, 2017.

2. Fig. 1D. Presumably MOSPD2 was identified from the 60 kDa band. Please make this clear. Was this the highest scoring hit (same question for VAPA/B from the 30kDa band) ie were these proteins identified in an unbiased way or were the authors more selective.
The three proteins listed in table Fig. 1C are the three top scored proteins identified by mass spectrometry, as stated in the figure legend. This identification was performed with an unbiased approach. The proteins were not identified from the gel after cutting out the bands; instead, they were directly analyzed by mass spectrometry from the liquid elution of the pull-down. To clarify this point, the materials and methods section was modified:
"For mass spectrometry analysis, eluted proteins were precipitated with trichloroacetic acid, and digested with Lys-C (Wako) and Trypsin (Promega). The peptides were then analysed using an Ultimate 3000 nano-RSLC (Thermo Scientific) coupled in line with an Orbitrap ELITE (Thermo Scientific)."
As stated in the Results section "Identified proteins were ranked based on their enrichment in the FFAT peptide sample over the control peptide sample, and on their MS/MS score." To be more precise, identified proteins were first filtered according to the ratio of their score in the FFAT sample over the Ctrl sample, in order to only consider proteins binding specifically the FFAT peptide and not the control peptide. After filtering, proteins were ranked based on their score: the three top scored proteins are in the table Fig. 1C.

3. Figure 2Aa.The colours in the figure, don't match the colours described in the legend (MOSPD2 is green in the figure, but the legend states it's orange).
The legend was corrected.

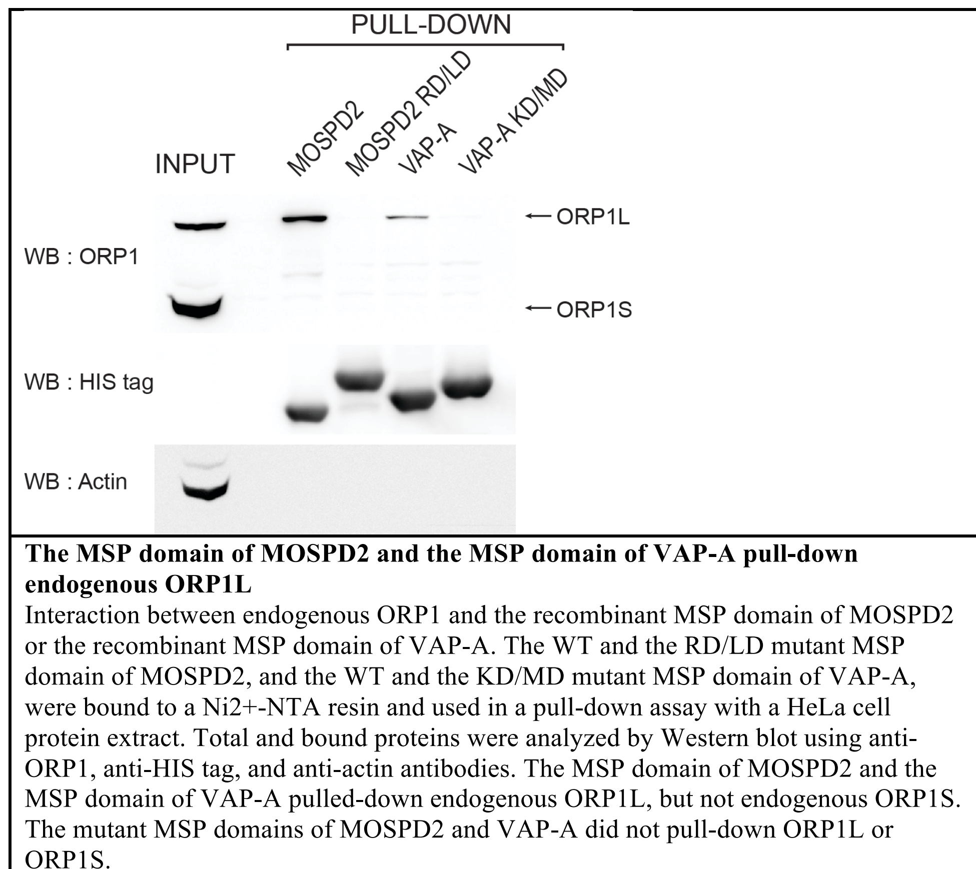
4. Figure 2Ca. Please comment on the size shifts for the mutants.
The mutation we introduced in the MSP domain of MOSPD2 replaces a basic (R404) and an aliphatic (L406) residue by acidic residues (D). These changes modify the global charge of the protein; it has been described in the literature that negative charges in proteins, by a mechanism of electrostatic repulsion of SDS, decrease SDS binding and thus reduce protein mobility (as described for instance in Shirai et al, JBC, 2008; Shi et al, Protein Sci, 2012; Guan et al, Sci Rep, 2015). We observed this phenomenon in MOSPD2 but also in VAP-A and in VAP-B (Fig. 2E). This is now indicated in the figure legend (Fig. 2E).
"Note that the mutant MSP domains of MOSPD2, VAP-A and VAP-B displayed a slowed migration, likely resulting from the negative charges introduced by the mutations."

5. Figure S1A. Does MOSPD2 have any obvious ER retention signals? Did the authors test whether the deltaC construct can also be relolcated upon coexpression with FFAT-proteins? This would be nice to know.

We looked for obvious ER retention signals in MOSPD2, but we could not find any. Yeast VAP proteins (Scs2 and Scs22) were shown to be retained in the ER by their transmembrane region (Loewen and Levine, JBC, 2005). Since these proteins are tail-anchored proteins resembling

MOSPD2, we posit that a similar mechanism is involved in ER retention of MOSPD2. Further work would be required to identify which part of MOSPD2 is indeed involved in its retention in the ER. In addition, as mentioned by the reviewer, we tested whether the delta C that we named MOSPD2 DTM protein was also relocated upon co-expression with FFAT proteins. These data are presented Figure EV1 C, D. Supporting the notion that the TM domain is indeed the ER anchor, MOSPD2 DTM protein was cytosolic and massively recruited onto STARD3 positive endosomes (Fig. EV1 C) and onto PTPIP51-positive mitochondria (Fig. EV1 D) in STARD3 and PTPIP51 expressing cells, respectively.

6. Ironically, the interaction between MOSPD2 and ORP1L is the least convincing at the IP (Figure 4C) and cellular (Fig. S3) level given that the FFAT motif used for identification is from an ORP!
We agree with this observation; the co-immunoprecipitation between MOSPD2 and ORP1L was repeated, but instead of using an anti-Flag antibody to detect ORP1L, we used an anti-ORP1L antibody. The anti-ORP1L antibody was more sensitive than the anti-Flag antibody. We believe that the IP is more convincing and it replaces the previous IP in panel 4C (Now Figure 5C).
We are presenting below another data which we hope will further convince the reviewer that ORP1L is a major partner for MOSPD2.



The MSP domain of MOSPD2 and the MSP domain of VAP-A pull-down endogenous ORP1L
Interaction between endogenous ORP1 and the recombinant MSP domain of MOSPD2 or the recombinant MSP domain of VAP-A. The WT and the RD/LD mutant MSP domain of MOSPD2, and the WT and the KD/MD mutant MSP domain of VAP-A, were bound to a Ni2+-NTA resin and used in a pull-down assay with a HeLa cell protein extract. Total and bound proteins were analyzed by Western blot using anti-ORP1, anti-HIS tag, and anti-actin antibodies. The MSP domain of MOSPD2 and the MSP domain of VAP-A pulled-down endogenous ORP1L, but not endogenous ORP1S. The mutant MSP domains of MOSPD2 and VAP-A did not pull-down ORP1L or ORP1S.

We performed a pull-down experiment using His-tagged recombinant wild-type and mutant MSP domains of MOSPD2 and VAP-A. Each recombinant protein was immobilized onto a NTA-Ni2+ resin and beads were then incubated with a HeLa cells protein extract. Proteins retained on the resin were eluted and analyzed by Western blot using an anti-ORP1 antibody. This antibody (Abcam; ab131165 EPR8646) recognizes both ORP1S and ORP1L, two variants of ORP1 generated by an alternative transcription initiation (Johansson et al, MBoC, 2002). Interestingly, ORP1L possesses a FFAT motif while ORP1S does not; accordingly, only ORP1L was shown to bind VAP proteins (Rocha et al, JCB, 2009). Our pull-down assay showed that the MSP domain of MOSPD2 and the MSP domain of VAP-A were able to pull-down ORP1L, and not ORP1S. Interestingly, the mutant MOSPD2 and VAP-A proteins unable to bind the FFAT motif did not pull-down ORP1L. These data confirm that the MSP domain of MOSPD2 is able to pull-down endogenous ORP1L.

Moreover, ORP1L was amongst MOSPD2 partners that we identified by mass spectrometry (See Fig. 5A and source data).

7. Figure 7. The immunostaining of endogenous MOSPD2 is not overly convincing as the residual staining is significant and worrying ER-like in the example shown. Did the authors test whether relocation of endogenous MOSPD2 is FFAT dependent? Please further quantify colocalization with correlation coefficients. Does the endogenous protein coIP with FFAT-proteins?
As mentioned by the reviewer, a residual staining is present when endogenous MOSPD2 is labeled in cells silenced with shRNAs. I just want to point out that this residual background is much weaker than MOSPD2 staining in control cells.
To confirm the specificity of the staining, as recommended by the reviewer, we quantified the colocalization of endogenous MOSPD2 with STARD3NL in cells expressing WT STARD3NL or a FFAT-mutant STARD3NL. These data are shown Fig. 8E-G. In the presence of STARD3NL, MOSPD2 accumulated around STARD3NL positive endosomes, while it remained evenly distributed in the ER in STARD3NL DFFAT expressing cells. Accordingly, STARD3NL and endogenous MOSPD2 signals were highly correlated, while STARD3NL DFFAT and endogenous MOSPD2 signals were not. We also tested whether the relocation of endogenous MOSPD2 was FFAT dependent in the case of other FFAT-containing proteins studied herein, namely STARD3, ORP1L, STARD11 and PTPIP51 (Fig EV6). Likewise, endogenous MOSPD2 was recruited around endosomes, Golgi and mitochondria in STARD3 (Fig. EV6A), ORP1L (Fig. EV6C), STARD11 (Fig. EV6E) and PTPIP51 (Fig. EV6G) expressing cells. In contrast, in cells expressing FFAT-deficient STARD3 (Fig. EV6B), ORP1L (Fig. EV6D), STARD11 (Fig. EV6F) or PTPIP51 (Fig. EV6H), endogenous MOSPD2 retained an even distribution within the ER.
In order to determine if endogenous MOSPD2 interacts with FFAT-containing proteins, we performed co-immunoprecipitation assays. WT and FFAT- mutant Flag-STARD3NL (Fig.5G) or STARD3 (Fig. EV3A) were expressed in HeLa cells and immunoprecipitated using anti-Flag and anti-STARD3 antibodies, respectively. Endogenous MOSPD2 was co-immunoprecipitated with Flag-STARD3NL (Fig.5G) and STARD3 (Fig. EV3A), and not with the FFAT-mutants Flag-STARD3NL DFFAT and STARD3 FA/YA. Thus, endogenous MOSPD2 interacts with FFAT-containing proteins.

8. Figure S2 legend has been duplicated.
The duplicated legend was removed.

9. LAMP1 is a late-endosome/lysosome marker.
Indeed, in Figure 5 (now Figure 6), in Figure S3 (now Figure EV4) and in Figure S4 (now Figure EV5), we specified that Lamp1 was used as a late endosome/lysosome marker.

10. Please add molecular mass markers to all blots.
As source data to this manuscript, we have added the uncropped Western blot images used in the different figures of the paper. The position of the molecular mass markers is shown on these images. In order not to overload the figures, we did not include this information in the main Figures.

Referee #3:

In this manuscript Di Mattia and colleagues reported the identification of a VAP-related protein, MOSPD2, that possibly plays a role in tethering between the ER and other organelles. Although the data presented are solid and prove that MOSPD2 binds FFAT motifs and can link lipid membranes in vitro, the relationship between MOSPD2 and VAPA/B in the context of the formation of membrane contact sites (MCS) has not been fully investigated.

1. To what extent MOSPD2 contributes to the formation of MCS between the ER and other organelles? Do MOSPD2 and VAPA/B independently, additively, or synergistically mediate membrane tethering? Are MCS disrupted upon depletion of only MOSPD2? What about VAPA/B and MOSPD2 double knockdown?

We believe that issues raised by the editor point 2 and 3 address these concerns. In brief, MOSPD2 recruits organelles by a FFAT-dependent mechanism. In vitro the isolated MSP domain of MOSPD2

is able to tether a FFAT-bound vesicle. Using TEM and stereology, ER-mitochondria are not significantly decreased by silencing either MOSPD2, VAP or both MOSPD2 and VAP (Fig. 9), while ER-endosome contacts were reduced by MOSPD2 depletion. No synergistic or additive effect were observed upon co-silencing of both MOSPD2 and VAP, supporting the idea that MOSPD2 has a tethering function independent of VAP. We show here that the MSP domains of both MOSPD2 and VAP have a similar affinity for FFAT peptide (Fig. 3), however we also show that steady state levels of VAP are superior (Fig. EV2) to those of MOSPD2. These findings suggest that the availability for a peculiar tether might guide the formation of specific contact sites. We also speculate that other mechanisms such as avidity (multiple FFAT motif on the same partner and protein dimerization and/or oligomerization) are modulating the tethering function of VAP and MOSPD2.

2. How about the specificity of target organelles? Are organelles linked by MOSPD2 the same as those by VAPA/B?

This is an interesting point, however it is difficult to address experimentally. We may answer at least in part to this issue by referring to the list of protein partners that were isolated using MOSPD2 as a bait and identified by mass spectrometry. The table presented Fig. 5A shows that proteins from endosomes, mitochondria and lipid droplets were pulled down, suggesting that at steady state MOSPD2 links at least these different organelles to the ER.

3.Does MOSPD2 compete with VAPA/B for binding to FFAT motif-containing proteins?

We addressed this point directly see ANSWER TO THE EDITOR POINT 4.

4.Is MOSPD2 co-localized with VAPA/B at MCS?

To address this question, we co-labeled endogenous VAP-A and endogenous MOSPD2 in cells expressing STARD3NL or the FFAT-defective mutant STARD3NL DFFAT. This data are now shown Fig. EV7. Interestingly, endogenous VAP-A was concentrated together with endogenous MOSPD2 in sub regions of the ER localized around STARD3NL positive endosomes (Fig. EV7B). In control cells or in cells expressing the mutant protein STARD3NL DFFAT, both VAP-A and MOSPD2 were not recruited around endosomes they remained evenly distributed in the ER (Fig. EV7 A and C). Thus, MOSPD2 and VAP are present in the same contact sites.

5.Regarding Fig. 1A: If the authors used this data as evidence that ER-mitochondria contacts are still present in VAPA/B-depleted cells, the data should be quantified.

Given the consensus that this panel was not necessary, we removed it and cited the literature. See answer to the editor point 1.

2nd Editorial Decision | 20 April 2018

Thank you for the submission of your revised manuscript to EMBO reports. I apologize for the delay in handling your manuscript but we have only recently received the last referee report. Please find the full set of reports copied below.

As you will see, the referees acknowledge that the manuscript has been significantly improved during the revision but the opinions remain somewhat divided. As you will see, referee 1 supports publication without further revision. Referee 2 asks for further clarification of the methodology and discrepancies with earlier literature. Referee 3 notes that the affinity measurements for VAPA/B, MOSPD2 and the FFAT motif do not directly address competitive binding for FFAT. While I certainly agree with this concern, I also note that these experiments, i.e., the identification of MOSPD2 binding partners and the competitive binding experiments were rated with lower priority and as not essential for the revision in my first decision letter. Therefore, I would like to invite you to address the remaining concerns from referee 2 and 3 in the text. Please discuss why the results obtained with VAPA/B depletion differ from published literature and please comment on your image analysis procedure. Moreover, you might want to carefully revisit the quantification of

VAPA/B and MOSPD2 protein levels.

Browsing through the manuscript myself, I noticed a few editorial things that we need before we can proceed with the acceptance of your study.

- Please reformat the references to match the numbered style of EMBO reports. You can download the respective EndNote file from our Guide to Authors:
https://drive.google.com/file/d/0BxFM9n2lEE5oOHM4d2xEbmpxN2c/view

- Please provide up to five keywords and a running title (max. 40 characters incl. spaces) on the first page of the manuscript.

- Please provide an Author Contribution section after the Acknowledgement section.

- Please move the Figure legends to the end of the manuscript, followed by a separate paragraph for EV figure legends.

- You have currently 8 EV figures. Expanded View figures will be displayed in the html version of the article in an expandable format but unfortunately, we can only accommodate up to 5 EV figures. Please choose 5 figures that you want to promote to Expanded View and provide the other figures in an Appendix. The Appendix includes a table of content on the first page with page numbers, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

- Callouts to figures: Currently you refer to Fig. 3I and J after you described Fig. 4. You might want to consider rearranging these panels so that the arrangement of the figures follows the flow of the text.

- Thank you for providing source data for all Western blots. This is very much appreciated. Could you please combine all files per figure into one pdf file, i.e., combine the files for Fig. 5 and EV2. Thank you.

- It is a precondition for publication in EMBO reports that authors agree to make all data freely available, where possible in an appropriate public database. In the case of mass spectrometry datasets, they should be deposited in a machine-readable format (e.g. mzML if possible) in one of the major public database, for example Pride (http://www.ebi.ac.uk/pride/) or PeptideAtlas(http://www.peptideatlas.org) and authors should follow the MIAPE recommendations (http://www.psidev.info/index.php?q=node/91).

Please deposit your data in one of these databases and provide the reference number in the manuscript in a separate Data availability section at the end of Material and Methods.

- Our data editors from Wiley have already inspected the Figure legends for completeness and accuracy. Please see their suggested changes in the attached Word file.

- Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large. For the larger image the height is variable. You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

We look forward to seeing a final version of your manuscript as soon as possible. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The authors have answered all of my questions and suggestions in the best possible way. I now find this paper highly suitable for EMBO Reports.

Referee #2:

The authors have responded well (if not verbosely!) with a substantial amount of data which largely support their original conclusions. In particular, the new EM data is welcomed. However, the quality of the images is suboptimal. And it is difficult to understand how the extent of organellar contact was derived when ER membranes was often hard to visualise. This is particularly pertinent when the results of VAPA/B depletion on ER-mitochondria and ER-endosome differ from published literature. The authors do not adequately address this in the Discussion. For example, the authors comment that the differences may be cell-type specific. However, the work of Eden et al used the same cell type (Hela) as in this study.

Referee #3:

The revised version of the manuscript by Di Mattia and colleagues, at least to my impression, has not been substantially improved. Although this is partly due to an unavoidable reason, i.e., depletion of VAPA/B, MOSPD2, or both had no obvious effects on membrane contact sites between the ER and other organelles, except that MOSPD2 silencing affected the ER-endosome contact, the authors have not directly responded to my comments. For example, regarding the competition experiment (Comment 3), this referee expected that the authors would examine whether the MSP domain of MOSPD2 competes with the MSP domain of VAPA/B with respect to the binding to FFAT motif-containing proteins (or the motif-peptide attached beads). Instead, the authors identified MOSPD2 partners by mass spectrometry and measured the binding constant for the FFAT-motif peptide by surface plasmon resonance. The results showed that MOSPD2 and VAPA/B share several common partners, and that MOSPD2 binds to the FFAT-motif peptide with an affinity similar to those of VAPA/B, but they do not straightforwardly answer the question. Although the authors showed that the amounts of VAPA, VAPB, and MOSPD2 in cells were differed by several orders of magnitude, this estimation appears to be flawed. Judging from the immunoblotting data shown in Figure EV2, the amounts of VAPA,VAPB, and MOSPD2 seem to be approximately 400-500, 200, and 30 fmol/μg, respectively, not 430, 15, and 2.2 fmol/μg, respectively, as the authors claimed. My estimation was consistent with the silver staining data of VAPA/B and MOSPD2 shown in Fig. 1B. This referee believes that the identification of a new protein that binds to FFAT-motif-containing proteins and can link liposomes is important and matches the "single message policy" of the journal. However, it may be difficult to accept this manuscript at this present form.

Minor points:
1. The authors identified organelles such as endosomes and indicated their positions in the linescan panels in Fig. 8D-F and Fig. EV6. How were their positions determined?

2. Typos:
- Fig. EV2A, B: Ve = 9.45 ml and = 9.22 ml, not 9,45 ml and 9,22, respectively ("point", not "comma".
- Legend to Fig. EV2: bovine serum albumin, not Bovine Serum Albumine. Similary, not Ovalbumine.

2nd Revision - authors' response 24 April 2018

Referee #1:

The authors have answered all of my questions and suggestions in the best possible way. I now find this paper highly suitable for EMBO Reports.

Referee #2:

The authors have responded well (if not verbosely!) with a substantial amount of data which largely support their original conclusions. In particular, the new EM data is welcomed. However, the quality of the images is suboptimal. And it is difficult to understand how the extent of organellar contact was derived when ER membranes was often hard to visualise. This is particularly pertinent when the results of VAPA/B depletion on ER-mitochondria and ER-endosome differ from published literature. The authors do not adequately address this in the Discussion. For example, the authors comment that the differences may be cell-type specific. However, the work of Eden et al used the same cell type (Hela) as in this study.

First, we have shortened some sentences in the text to limit the verbosity. Second, we have also better explained the way contacts are measured in this study. This will probably explain why our image may appear of suboptimal quality to this reviewer. Indeed, we used stereology to quantify the length of apposed membranes from distinct organelles. To be accurate, the ultrastructure must be preserved in order to avoid organelle deformations. To date, EM sample preparation requires to compromise between preservation and contrast. We chose a protocol suitable for quantification of contacts which preserves the sample ultrastructure: we performed high pressure freezing and freeze substitution of the samples with minimal heavy metal amounts (EMBL electron microscopy facility; Heidelberg). This enhanced preservation of the sample, which is mandatory for contact site quantification, is at the cost of contrast. However, the endoplasmic reticulum membrane is readily visible and contacts are quantifiable.

Third, concerning the difference between our results and the published literature, we have modified the text to be more precise. Actually, it was misleading to say that our results differ from the published literature because these studies were not comparable. Stoica et al., (Nat Com 2014) measured ER-mitochondria contacts in NSC-34 motoneuron-like cells. They showed that loss of VAPB result in a 30% decrease of ER-mitochondria contacts in these highly specialized cells. It is known that motoneurons are dependent on VAP since mutations in VAPB are causing loss of these cells in Amyotrophic Lateral Sclerosis (ALS) a fatal neurodegenerative disease. Eden et al (Dev Cell 2016) studied ER-endosome contacts in the context of EGF signaling. In their experiments they performed serum starvation followed by an acute treatment with EGF, both treatments are known to affect the endocytic pathway. In addition, they followed the fate of two kind of endosomes: EGFR-containing endosomes and non EGFR-containing endosomes. They showed that only one of these two sub-populations is affected by VAPA/VAPB silencing (contacts decreased by ~50%). They did not quantify the prevalence of this kind of endosomes over the other one, therefore it is not possible to know the effect of VAP silencing on all endosomes. In our experiment, we did not treat cells with EGF and we quantified all endosomes without making this distinction. Moreover Eden et al., did not quantify the length of membrane contact but their frequency. To conclude we modified the discussion and pointed out that while we did not find a significant effect of VAP depletion on ER-mitochondria and ER-endosome contacts, other studies using specialized cells and different experimental setting found that VAP loss reduced ER-mitochondria and ER-endosomes contacts.

"In our experiments, VAP loss was not associated with a significant decrease of ER-mitochondria or ER-endosome contacts. However, other studies that have looked at specific contacts, such as those occurring during EGF signaling, found that ER/EGFR-endosome contacts were unaffected by the loss of VAPs, while the ER/non-EGFR-endosome contacts were impaired by 50% [24]. In addition, in highly specialized cells like motoneurons, Stoica et al showed a decrease of ER-mitochondria contacts after VAP-B silencing [16]. These findings and our results probably reveal a cell type- and context-dependent requirement of VAP proteins in MCS formation."

Referee #3:

The revised version of the manuscript by Di Mattia and colleagues, at least to my impression, has not been substantially improved. Although this is partly due to an unavoidable reason, i.e., depletion of VAPA/B, MOSPD2, or both had no obvious effects on membrane contact sites between the ER and other organelles, except that MOSPD2 silencing affected the ER-endosome contact, the authors have not directly responded to my comments. For example, regarding the competition experiment (Comment 3), this referee expected that the authors would examine whether the MSP domain of MOSPD2 competes with the MSP domain of VAPA/B with respect to the binding to FFAT motif-containing proteins (or the motif-peptide attached beads). Instead, the authors identified MOSPD2 partners by mass spectrometry and measured the binding constant for the FFAT-motif peptide by surface plasmon resonance. The results showed that MOSPD2 and VAPA/B share several common partners, and that MOSPD2 binds to the FFAT-motif peptide with an affinity similar to those of VAPA/B, but they do not straightforwardly answer the question. Although the authors showed that the amounts of VAPA, VAPB, and MOSPD2 in cells were differed by several orders of magnitude, this estimation appears to be flawed. Judging from the immunoblotting data shown in Figure EV2, the amounts of VAPA,VAPB, and MOSPD2 seem to be approximately 400-500, 200, and 30

fmol/µg, respectively, not 430, 15, and 2.2 fmol/µg, respectively, as the authors claimed. My estimation was consistent with the silver staining data of VAPA/B and MOSPD2 shown in Fig. 1B.

This referee believes that the identification of a new protein that binds to FFAT-motif-containing proteins and can link liposomes is important and matches the "single message policy" of the journal. However, it may be difficult to accept this manuscript at this present form.

This referee noticed a problem with the quantification of VAPA, VAPB and MOSPD2 amounts in HeLa cells. Indeed, we wrongly labeled the amount of recombinant VAP-A protein in panel E (now Appendix Figure S1). The quantities of proteins were not 10150, 610, 365, 220 and 110 fmoles, but 10150, 6090, 3650, 2190 and 1100 fmoles. This labelling mistake does not affect the result of the quantification. In addition, we did not indicate the amount of total whole protein extract loaded. This has been corrected, please note that the amount of protein extract loaded on the gel was 13 µg (MOSPD2, panel D), 10 µg (VAP-A panel E) and 10 µg (VAP-B panel F).

Minor points:
1. The authors identified organelles such as endosomes and indicated their positions in the linescan panels in Fig. 8D-F and Fig. EV6. How were their positions determined?
The position of organelles on the linescan was based on the fluorescent signal of STARD3, STARD3NL, STARD11 and PTPIP51 which were previously described to be localized in endosomes (STARD3, STARD3NL, ORP1L), Golgi (STARD11) and mitochondria (PTPIP51). Moreover, these localizations were confirmed in our study (see Fig. 6, Fig 7, Fig. EV3).

2. Typos:

- *Fig. EV2A, B: Ve = 9.45 ml and = 9.22 ml, not 9,45 ml and 9,22, respectively ("point", not "comma".*
- *Legend to Fig. EV2: bovine serum albumin, not Bovine Serum Albumine. Similary, not Ovalbumine.*

These typos were corrected.

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Corresponding Author Name: Fabien Alpy
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2017-45453

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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

A- Figures
1. Data
The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

- ➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- ➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- ➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
- ➔ definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

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B- Statistics and general methods

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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size for the studies was chosen according to previous studies in the same area of research. In general 2 to 4 independent experiments were performed.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No sample was excluded
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.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	The test used for each data set is specified in the figure legend.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. Non-parametric tests were used.
Is there an estimate of variation within each group of data?	Yes: see figures (and their legend) and error bars.
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

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).	Antibodies used are described in the materials and methods section; the description includes a citation for non-commercial antibodies and a catalog/clone number for commercial antibodies.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HeLa cells originate from Dr Walter Schaffner laboratory (Switzerland) and were recently authentified by LGC Standards as identical to ATCC cell line CCL-2 (HeLa). 293T cells originate from Dr Christof Niehrs laboratory (Germany) and were recently authentified by LGC Standards as identical to ATCC cell line CRL-3216 (293T). Cell lines are regularly tested for mycoplasma contamination in the IGBMC cell culture facility.

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D- Animal Models

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

E- Human Subjects

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

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
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).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

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