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FFAT motif phosphorylation controls formation and lipid transfer function of inter-organelle contacts

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1st Editorial Decision 8th Jan 2020

Thank you for submitting your manuscript (EMBOJ-2019-104369) to The EMBO Journal. Please accept my apologies for the delay in getting back to you with our decision due to the recent seasonal holidays. I have now read your study carefully and discussed the work with the other members of the editorial team; I regret to inform you that we have decided not to pursue publication of this manuscript.

We appreciate that you identify a non-conventional FFAT motif in endosomal STARD3 and that phosphorylation of FFAT on Ser209 is required for its binding to the MSP domain of ER-associated VAP-A and MOSPD2, and for recapitulating the formation ER-endosome contact sites in vitro. Furthermore, you solve the crystal structure of the MSP domain of VAP and MOSPD2 in complex with phosphorylated FFAT, identify and validate a set of proteins harbouring a non-conventional FFAT motif.

We recognize the high quality of your work and that you are the first to identify a non-conventional FFAT motif, the phosphorylation of which may control ER-endosome contact sites. However, we would require further insight into how FFAT phosphorylation is regulated in cells and the kinase(s)/phosphatase(s) involved. Consequently, we find that your study, in its current form, is not a strong candidate for publication in The EMBO Journal and we have decided not to send it out for in-depth review. Nevertheless, given the clear relevance of your data to the more immediate field, I would be happy to reconsider your study if you could provide conclusive data on how FFAT phosphorylation is controlled and regulates contact-site formation and sterol transfer between the ER and the endosomes in cells.

I am aware that this is a very competitive field and understand that you might prefer to submit your work as is elsewhere. In this case, I find that your study would be a good candidate for our sister journal EMBO reports, which focuses on the novelty of the findings, rather than on molecular mechanism(s). If you are interested in this option, I would be happy to discuss your manuscript with my colleague Martina Rembold.

I thank you for giving us the opportunity to consider this manuscript and look forward to hearing from you.

Please find our manuscript entitled “Phosphorylation switches on inter-organelle contacts” to be considered for publication in The EMBO Journal. This manuscript addresses the question of how inter-organelle membrane contact sites are regulated.

This is a revised version of the manuscript we had submitted early this year. We took your suggestions into consideration and we added experiments to address your concerns. This manuscript has a new figure (Fig. 6) which describes *in situ* cholesterol labeling experiments showing that STARD3 phosphorylation regulates cholesterol transport in cells. In my previous correspondence with you, I had proposed to include experiments on Kv2.1 in the manuscript. These experiments were ongoing before the lockdown but are now halted; hopefully they will be resumed in the following weeks/months. Meanwhile, I thought it would be worth contacting you to know if you are willing to send the manuscript for in-depth review.

Moreover, your comments made us realize that the general implications of this work in the field of inter-organelle membrane contacts were not clear enough and underemphasized. To circumvent this issue, the paper was rewritten.

I would like to underline more specifically the following points:

1.

A central question in the field of cell biology is how the assembly of membrane contact sites can be done and undone. This remains poorly documented. VAP proteins are pivotal in the formation of major contact sites between the ER and others organelles (endosome, Golgi...) as well as the plasma membrane, by recruiting various FFAT-containing proteins. Various proteins were known to have a conventional FFAT recognized by VAP proteins (see review Murphy and Levine, BBA, 2016). Here, we uncovered that about the same number of proteins which include STARD3, FIP200, MIGA2, PTPIP51, Kv2.1 and Kv2.2 contain a non-conventional FFAT motif (termed Phospho-FFAT) that depends on phosphorylation to interact with VAPs and MOSPD2, a newly discovered VAP-related tether protein. To do so, we built a predictive tool to identify these proteins but also other potential VAPs/MOSPD2 interactors. Thus, our work extends the list of validated or potential partners of VAPs/MOSPD2 proteins while more precisely defining their mode of binding.

2. Our work provides novel atomistic insights into the formation of contact sites. We solved the structure of the MSP domain of VAP bound to the Phospho-FFAT motif. We also solved the structure of the MSP domain of the FFAT-recognizing protein MOSPD2, and we unveiled its mode of interaction with conventional FFAT and Phospho-FFAT motif. This part of work is also important as it authenticates MOSPD2 as a third VAP protein interacting with FFATs, and Phospho-FFATs.

3. To establish the biological significance of Phospho-FFATs, we studied the lipid transport STARD3 protein as a prototypical case to show that *in vitro* and *in vivo* phosphorylation of this protein is required for its interaction with VAP-A/VAP-B/MOSPD2, the formation of ER-endosome contacts, and cholesterol transport towards endosomes.

To summarize, we present findings of great interest to the audience of The EMBO Journal. We believe that our manuscript will be read by a variety of scientists working in the fields of cell biology, membrane biology, lipid metabolism, protein/membrane interaction and structural biochemistry. It is the case of our previous papers on this subject published in The Journal of Science (Alpy et al, 2013, cited 149 times Google scholar), The EMBO Journal (Wilhelm et al, 2017; cited 81 times) and EMBO reports (Di Mattia et al, 2018; cited 21 times).

1st Revision - Editorial Decision 6th May 2020

Thank you for submitting your manuscript entitled "Phosphorylation switches on inter-organelle contacts" [EMBOJ-2020-105120] to The EMBO Journal. Your study has been sent to three reviewers for evaluation, whose reports are enclosed below.

As you can see, the referees consider the work potentially interesting. However, they also raise few criticisms that need to be addressed before they can support publication in The EMBO Journal.

Given the overall interest of your study, I am pleased to invite submission of a revised manuscript as indicated in the referee's reports. I would like to point it out that addressing all the referees' points in a conclusive manner will be essential for publication in The EMBO Journal, as well as a strong support from the referees.

Please note that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage.

When preparing your letter of response to the referees' comments, bear in mind that this will form part of the Review Process File and therefore will be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process.

Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see http://msb.embopress.org/authorguide#dataavailability). Remember to provide a reviewer password if the datasets are not yet public.

We usually expect to receive revised manuscripts within three months of the first decision. We are aware that many laboratories cannot function at full capacity during the current COVID-19/SARS-CoV-2 pandemic and may relax this deadline. Also, we can extend our 'scooping protection policy' to cover the period required for a full revision to address all of the referees' points. Please inform us as soon as a paper with related content published elsewhere.

Thank you again for the opportunity to consider this work for publication, and please feel free to contact me with any questions about submission of the revised manuscript to The EMBO Journal.

Referee #1:

MCSs play an important role in interorganelle lipid exchange, organelle biogenesis, and dynamics. VAP-A, VAP-B, and MOSPD2 are ER proteins involved in membrane tethering: these proteins have an MSP domain that associates with FFAT motifs of proteins on the opposing membrane. In the current manuscript, Di Mattia et al. describe a new mechanism for assembly/disassembly of ER-

endosomes MCSs and sterol transfer. Through an in-silico approach, they identified a non-canonical FFAT domain in several known binding partners of VAP-A, VAP-B, and MOSPD2. This domain has a residue that needs to be phosphorylated in order to bind the MSP domain of the ER-bridging proteins. Here, the authors build on their previous findings, where they identified STARD3 as an ER-endosome tether and cholesterol transfer protein, through its contact with VAP.
The main novelty of this manuscript is the description of the reversible binding mechanism of STARD3 to ER receptors. They also solved the crystal structure of phosphorylated STARD3 with VAP-A and MOSPD2 and quantified the binding affinities via surface plasmon resonance and pull-down experiments. The authors show that a specific modification (phosphorylation) in the FFAT domain of STARD3 regulates ER-late endosome contact formation and cholesterol transfer. The manuscript is well structured and uses a wide array of in-vitro techniques complemented with immunofluorescence in cells. The findings are of high interest to the organelle biology field. The mechanisms involved in the formation of MCSs and how these contacts are maintained while organelles traffic are still big questions that this work helps to answer. The results are confirmed with the appropriate controls and mutants, making it suitable for publication in the EMBO journal.
I have minor concerns about the functional meaning of the findings:
-Which is the function of STARD3 that needs the ER-endosome contact to be reversible vs. a constitutive binding? Why does phosphorylation give this additional function and not just enhance the sterol transfer like other proteins (e.g. STARD11)?
-Fig 4 and Fig7: What is the localization of STARD3 phospho-mutants in a STARD3 knock-out background? Endogenous STARD3 is present and can be competing for the VAP/MOSPD2 binding site, therefore pushing phospho-STARD3 and changing its localization.
-Fig 6: fluorescence microscopy quantification is not convincing to show sterol accumulation:
o What is the late endosome mask and how was the threshold used? Please show.
o CD63 staining is not specific to endosomes on C and D, with an increased background. How was background subtracted? How did you account for fluorescence bleed through? A more specific marker could be used.
o B, C: STARD3 and STARD3 S209A localization in C and in Fig4 D is different. Here it is more homogeneous, bigger aggregates. Is it due to the use of an inducible system? Can that affect the localization and filipin recruitment?
-The predictive algorithm suggests that 10% of the 20,000+ proteins they looked at have a phospho FFAT motif. This seems a very high hit rate for these algorithms and suggests that more stringent criteria for evaluation are necessary. Particularly, since it seems unlikely that all 2,000+ proteins will be able to by bind MSP domains.

Referee #2:

In this study, the authors investigate a mechanism by which membrane contact sites between the ER and other organelles could be regulated. The ER possesses three major tethering/receptor proteins (VAPA, VAPB, and MOSPD2) that are involved in the formation of contact sites with most organelles. These three proteins contain an MSP domain allowing them to bind proteins with a motif named FFAT. The FFAT-motif containing proteins are diverse in their functions, and they include lipid transporters and ion transporters and apposition tethering proteins from almost all other organelles. Since these FFAT motif containing proteins grossly outnumber the ER tethering proteins, it suggests that interaction between the MSP domain and FFAT-motifs is likely tightly regulated. In this manuscript, the authors demonstrate how phosphorylation of the FFAT-motif may regulate binding with the MSP domain proteins. The authors identified proteins with a non-conventional FFAT motif with a serine or threonine at the 4th position of the motif. One such

protein in the lipid transporter STARD3. Using various biochemical and cellular techniques, the authors demonstrate that the phosphorylation of the 4th serine residue is critical for STARD3 binding to the MSP domain proteins. Further, using both structural analysis and in vitro binding assays, the authors show a difference in the phospho-FFAT motif of STARD3 binding to MOSPD2 compared to binding to VAPA or VAPB.

This manuscript is a very robust study that provides a novel mechanism of the regulation of ER contact sites, which would be of great interest to a broad audience and particularly to the readers of EMBOJ. The authors used an impressive combination of a wide range of experimental techniques to establish the importance of the phosphorylation of the Phospho-FFAT motif of STARD3 in its interaction with VAPA/B and MOSPD2, as a prototype to study Phospho-FFAT motif and their regulation by phosphorylation. The finds presented in this manuscript add a new layer to our understanding of FFAT motifs and why they could be so variable. They also highlight differences between MSP domain-containing proteins (VAPs and MOSPD2). Moreover, structural and bio-informatics data provided with this study offers a new resource for the membrane contact site field. Overall, the manuscript is well written, and the authors are describing their experiments very clearly. The data are pretty convincing, and I have only some minor points that I think should be addressed.

Minor points:
• Fig 1C, Fig2C, and Fig7B: The negative control used should be stated in the figure legends (it is only in the main text).

• Fig 1E: the table should read "Phospho-FFAT" score and position and not "FFAT" score and position.

• Fig4: What are the FFAT (and/or Phospho-FFAT) score of the STARD3 mutants generated? Do the mutations increase or decrease this score? This should be stated in the legends and/or text.

• Fig4: A STARD3 P210A construct with only the proline mutated in Alanine (without mutating the Serine) should be used to control that this mutation does not, by itself, promote the interaction with VAPA (or VAPB) either by Co-IP or confocal microscopy.

• In Fig S6 (Fluorescence microscopy) or Fig 4C (Co-Ip), it seems that the STARD3 mutant S209D can interact with VAPB (although much less than WT or S209A/P210A) but not with VAPA (Fig4A and H). Can this small interaction be explained by structural differences in the MSP domain of VAPB compared to VAPA?

• Fig5: In the main text the authors conclude that "Thus, in a complete in vitro defined system, the sterol transport function of STARD3 is turned on by phosphorylation of its FFAT motif". In other words, the authors suggest that the phosphorylation of its FFAT motif directly regulates the lipid transfer ability of STARD3. However, this is an over-interpretation of their data. Taken together with their binding data, more likely interpretation of the liposome data is that S209 phosphorylation in STARD3 is required to establishment contact between the liposome via its being with VAP. And this close contact between these liposomes allows STARD3 to transfer DHE between them. To demonstrate whether this phosphorylation directly activates the sterol transfer ability of STARD3 the authors should test the DHE transfer in the presence of artificial tethers that would mediate the contact of liposomes between them independently of STARD3-VAP and with the FFAT domain of STARD3 phosphorylated or not (and with or without VAP). Otherwise, the authors should modify this statement in the main text.

• Fig. 5D: In the DHE transport experiment, the authors hypothesize that the decrease in the FRET signal is due to the transfer of DHE from LB to LA by pS209 cSTD3. However, similar results can occur if the two liposomes fuse. The authors need to rule out that fusion is not occurring between the liposomes.

• Fig6: Does the overexpression of the phosphomimetic S209D/P210A promotes the accumulation of cholesterol in late endosomes? According to Fig4E, this mutant can interact with VAPA to establish ER endosome contact site. Therefore, the authors should include the S209D/P210A mutant in this assay to examine whether it acts as a constitutive active motif similar to the Conv-FFAT.

• Fig 4 A, H and Fig7 F: It seems that the phosphomimetic mutants (S209D/P210A for VAPA and S209D for MOSPD2) do not interact as strongly as the wild-type STARD3 as evaluated by co-IP or colocalization. It is counter-intuitive as the phosphomimetic mutations should block STARD3 in an "ON" state for the interaction with VAPA or MOSPD2. Could the authors comment on that? Can this be explained by structural data showing the limitations of phosphomimetic mutations in this case?

• Fig 4D, 6C and S6C: the overexpression of STARD3 S209A appear to promote the re-localization of Late endosomes to the perinuclear region. Is this true? Is it linked to a known role of STARD3 in late endosomes trafficking?

• In a perfect conventional FFAT motif (FFAT score = 0), if the Asp at the fourth position is replaced by a Ser is there still an interaction with MSP domain, or does it require the phosphorylation of the Serine (for example in a pull-down assay as Fig1C or 2C)?

Referee #3:

This study by Di Mattia et al. identifies and characterizes special type of FFAT motifs in the human proteome that are regulated by phosphorylation. Conventional FFAT (two phenylalanines in an acidic track) motifs provide interaction of proteins with the ER localized VAP-A and VAP-B proteins as well as with the recently described MOSPD2 protein at ER-to other organelle membrane contact sites. This study used an in-silico approach to identify FFAT motifs in which phosphorylatable serine or threonine residues substitute for the conserved D/E residues in position 4 of conventional FFAT domains. This analysis identified 110 proteins that scored high with these analyses. Two of these proteins, Kv2 potassium channels have already been described with such a phospho-regulated FFAT-like motif.
The authors then selected one of the proteins, STARD3 for an extensive analysis of its FFAT-like domain using an impressive repertoire of assays. These included peptide binding to recombinant MSP domains (the domains within the VAP and MOSPD2 proteins that interact with FFAT motifs) and determination of the X-ray structures of the human VAP-A and MOSPD2 MSP domains with the bound STARD3 FFAT peptide. Moreover, the Authors analyzed the role of the phospho-FFAT motif of STARD3 in its interaction with the VAP and MOPSD2 proteins in intact cells, and the role of this interaction in cholesterol transport both analyzed in intact cells and in an in vitro liposome transfer assay. Collectively, these studies convincingly showed that phosphorylation of the key serine residue (S209) in STARD3 is critical for its function as a sterol transfer protein between the ER and endosomes.

The topic of non-vesicular lipid transport between the ER and other organelles has received enormous attention in recent years because of its importance in cellular lipid metabolism and membrane biology with ample connection to human diseases. FFAT motifs found in many lipid transfer proteins have been well documented as a critical structural element for the proper function of these proteins. The discovery that about equal number of phospho-regulated FFAT motifs exist in the human genome is a very important step in this field that will allow future studies to explore their importance in the many proteins identified in this study.

The experiments featured in this work are very well-done and well-documented, convincingly supporting the conclusions. If anything, the Authors present more data and supplementary data than would be suffcient to make their case. However, all of these experiments are strong and well-organized and should be kept for the interested reader.

I have very few comments that might be useful to address to make the paper more accessible for the average reader.

1. The authors should describe better the various screening algorithms. For example, it is not clear what the score 1 and score 2 means in Tables S1 and S2?
2. Please, define DHE (dehydroergosterol) and DNS-PE (dansyl)-PE in the text and in Figure legends (Fig. 5)
3. Also, please describe the principle of the liposome assay (FRET) and what the curves mean in Fig 5D.
4. Was the binding of phosphomimetic pS209 peptides to VAP-MSP domains tested in the recombinant assays? Convincingly it was not sufficient in the cellular assays using the full-length protein.
5. What are the predictions for binding of the phosphor-FFAT of STARD3 to the ALS mutant MSP domain of VAP-B(P56S)? Was it tested by any chance?
6. Were there other serines phosphorylated in the phospho-cSTD3 prep from E.Coli or only P209?
7. Does the MOSPD2 MSP domain pFFAT of STARD3 peptide interaction in the X-ray structure explain why the phosphomimetic mutant binds here but not in the VAP-A MSP domain?
8. Table S2 has 110 (line 18 on page 5) or 109 (line 29, page 5) partners. Should not they be the same?

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Please find a modified version of our revised manuscript entitled "Phosphorylation switches on inter-organelle contacts" (EMBOJ-2020-105120).

We would like to thank the reviewers for their careful evaluation of the manuscript. The referees' concerns (boxed text) are discussed in detail below. The corresponding changes that we have made in the text are also indicated, as well as being printed in red in the revised manuscript. We feel that these changes improve the manuscript. Of note, we performed the cholesterol accumulation experiment using another late endosome/lysosome marker to answer Reviewer 1's concerns. We added a supplementary figure to describe the image analysis workflow we used to quantify filipin fluorescence in endosomes. Additionally, we added several control experiments (peptide binding experiments; liposome fusion assay) which are now described in the manuscript.

Point by point answers to the reviewers

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Referee #1:

MCSs play an important role in interorganelle lipid exchange, organelle biogenesis, and dynamics. VAP-A, VAP-B, and MOSPD2 are ER proteins involved in membrane tethering: these proteins have an MSP domain that associates with FFAT motifs of proteins on the opposing membrane. In the current manuscript, Di Mattia et al. describe a new mechanism for assembly/disassembly of ER-endosomes MCSs and sterol transfer. Through an in-silico approach, they identified a non-canonical FFAT domain in several known binding partners of VAP-A, VAP-B, and MOSPD2. This domain has a residue that needs to be phosphorylated in order to bind the MSP domain of the ER-bridging proteins. Here, the authors build on their previous findings, where they identified STARD3 as an ER-endosome tether and cholesterol transfer protein, through its contact with VAP.

The main novelty of this manuscript is the description of the reversible binding mechanism of STARD3 to ER receptors. They also solved the crystal structure of phosphorylated STARD3 with VAP-A and MOSPD2 and quantified the binding affinities via surface plasmon resonance and pull-down experiments. The authors show that a specific modification (phosphorylation) in the FFAT domain of

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STARD3 regulates ER-late endosome contact formation and cholesterol transfer. The manuscript is well structured and uses a wide array of in-vitro techniques complemented with immunofluorescence in cells. The findings are of high interest to the organelle biology field. The mechanisms involved in the formation of MCSs and how these contacts are maintained while organelles traffic are still big questions that this work helps to answer. The results are confirmed with the appropriate controls and mutants, making it suitable for publication in the EMBO journal.

I have minor concerns about the functional meaning of the findings:

-Which is the function of STARD3 that needs the ER-endosome contact to be reversible vs. a constitutive binding? Why does phosphorylation give this additional function and not just enhance the sterol transfer like other proteins (e.g. STARD11)?.

We would like to thank the Reviewer for her/his very highly positive comments on our manuscript.

Live imaging showed that the interaction between organelles is in most cases a dynamic process; organelles associate and dissociate over time (Valm et al, Nature, 2017). It is the case for ER-endosome contacts, indicating that the tethering machineries between these organelles are by essence reversible.

The need for a reversible interaction between STARD3 and VAP lies in the primary structure and in the sterol transport function of STARD3. STARD3 is a membrane-anchored protein: it is therefore constitutively associated with the membrane of late endosomes. By interacting with VAPs/MOSPD2, which are also membrane-anchored proteins, STARD3 creates ER-endosome contacts. In this context, sterols flow towards endosomes and they accumulate cholesterol at the expense of other membranes. Intracellular accumulation and disruption of the physiological distribution of cholesterol is detrimental. A reversible association enables the cell to adapt cholesterol transport according to demand. STARD3, as an ER-endosome tether, and at the same time an integral endosomal protein and a sterol transporter, is quite unique; most tethers are not anchored in membranes and they have other ways to dissociate from their bound organelle. This notion is illustrated in the discussion where we describe the sterol transporter OSBP, which is targeted to the Golgi by the affinity of its PH domain for PI(4)P (phosphatidylinositol 4-phosphate). There, OSBP uses a conventional FFAT motif to bind VAP and acts as an ER-Golgi tether. In the case of OSBP, it is the regulation of PI(4)P levels and the activity of Arf1 which, by affecting OSBP's ability to bind to the Golgi, regulate ER-Golgi contacts. In the case of STARD3, the association with the membrane cannot be regulated owing to the very nature of its transmembrane domain, which suggests that the regulation of STARD3-VAPs/MOSPD2 interaction by phosphorylation is the unique way to mitigate ER-endosome contacts created by STARD3. In this work we have made the striking observation that the proteins containing a single phospho-FFAT motif which we identified and tested (MIGA2, PTPIP51, Kv2.1 and Kv2.2) are anchored to the membrane as it is the case for STARD3. These examples support the idea that the phosphorylation of Phospho-FFATs provides a good way to regulate the association of integral membrane proteins with VAPs/MOSPD2. This hypothesis is presented in the discussion.

Another general consequence of the presence of Phospho-FFAT is that proteins possessing this type of motif may have two distinct functions, one in a complex with VAP (when phosphorylated) and another one without VAP (when non-phosphorylated). A recent paper (Freyre et al, Molecular Cell, 2019) described the role of the mitochondrial MIGA2 protein in the formation of ER-mitochondria and lipid droplet (LD)-mitochondria contacts. The authors described that MIGA2 forms a complex with VAP to create ER-mitochondria contact sites, but that MIGA2 is not associated with VAP to create LD-mitochondria contacts. By identifying a Phospho-FFAT in MIGA2, our work provides clues to understand the molecular mechanism of this observation. It is more than probable that the Phospho-FFAT of MIGA2 is phosphorylated in ER-mitochondria contacts and unphosphorylated in LD-mitochondria contact, and thus that phosphorylation controls the balance between these contacts.

- -Fig 4 and Fig7: What is the localization of STARD3 phospho-mutants in a STARD3 knock-out background? Endogenous STARD3 is present and can be competing for the VAP/MOSPD2 binding site, therefore pushing phospho-STARD3 and changing its localization.

To answer this question, we silenced STARD3 using a siRNA strategy in HeLa cells, and we transfected WT and mutant STARD3 using a cDNA with silent mutations making it insensitive to siRNAs. This strategy allowed us to express STARD3 (WT and mutants) in cells devoid of endogenous protein, and to assess the localization of the protein. We did not observe a different localization of STARD3 (WT, S209A, S209D/P210A) in the absence of endogenous STARD3. Moreover, we analyzed STARD3/VAP co-localization using Pearson correlation coefficient in cells treated with a control siRNA or a siRNA targeting STARD3, and the co-localization was similar in these two conditions. Thus, endogenous STARD3 does not affect the localization of STARD3 phospho-mutants (see new figure panel; Figure EV4 F).

-Fig 6: fluorescence microscopy quantification is not convincing to show sterol accumulation:

o What is the late endosome mask and how was the threshold used? Please show.

o CD63 staining is not specific to endosomes on C and D, with an increased background. How was background subtracted? How did you account for fluorescence bleed through? A more specific marker could be used.

o B, C: STARD3 and STARD3 S209A localization in C and in Fig4 D is different. Here it is more homogeneous, bigger aggregates. Is it due to the use of an inducible system? Can that affect the localization and filipin recruitment?

o What is the late endosome mask and how was the threshold used? Please show.

The fluorescence microscopy quantification workflow is now explained in a supplementary figure (Appendix Figure S4). Images of randomly chosen cells were acquired using the same parameters with a confocal microscope equipped with a 355 nm laser beam. Image analysis was performed with Fiji using a macro whose code is deposited on Github:

https://github.com/fabienalpy/Fiji_Filipin_Staining_Quantification_Endosome_Mask

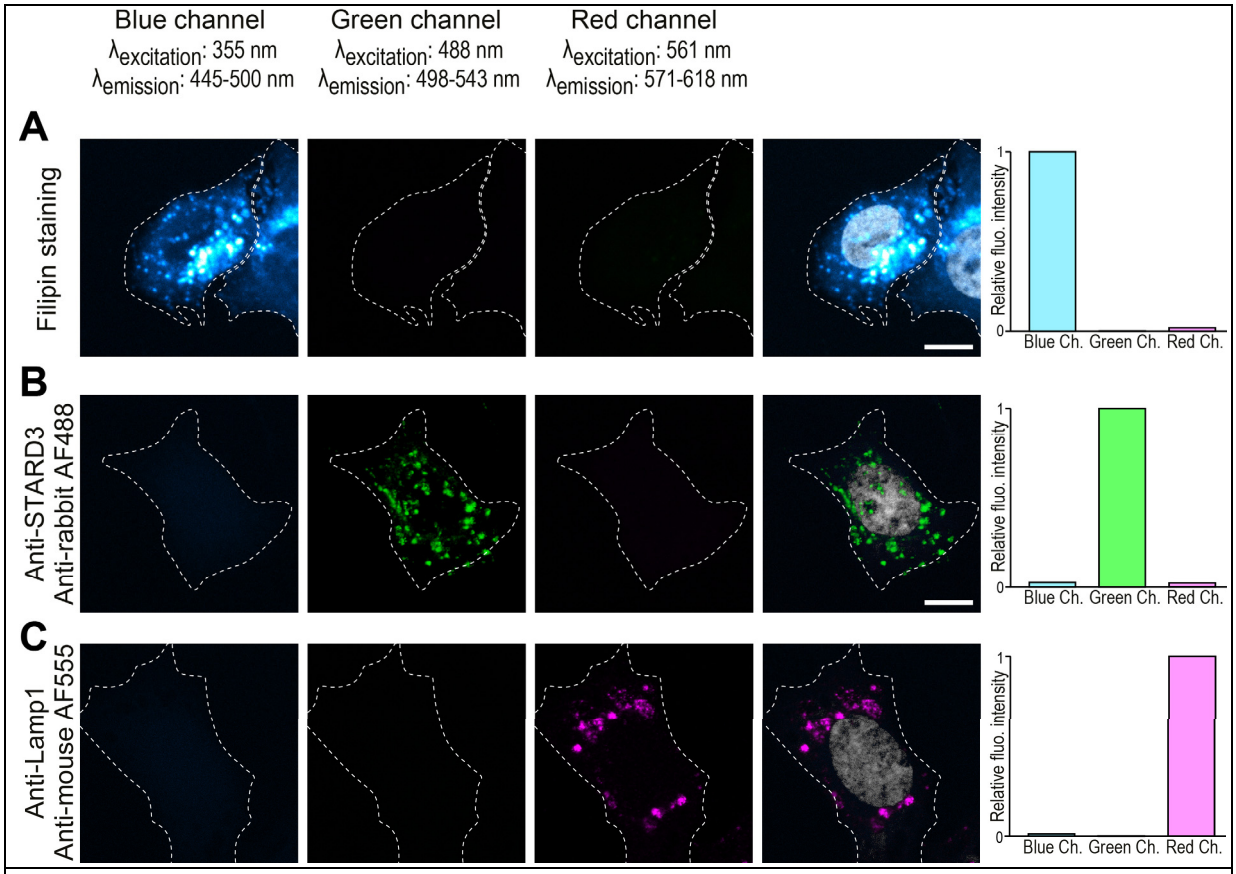
Experimental details are now indicated in the Materials and Methods section.

In order to segment late endosome/lysosome on the images, we performed a Gaussian blur on Lamp1 images, followed by an IsoData thresholding. This generated a mask corresponding to Lamp1-positive structures. This mask was then used to restrict the measure of filipin staining intensity to Lamp1-positive vesicles. Cell contours were manually segmented and the mean pixel intensity in the Lamp1-mask was measured for individual cells.

o CD63 staining is not specific to endosomes on C and D, with an increased background. How was background subtracted? How did you account for fluorescence bleed through? A more specific marker could be used.

As noted by this reviewer, the CD63 staining was not ideal, as the antibody we used gave some background. To circumvent this issue, the experiment was repeated with another late endosomes/lysosomes marker, Lamp1. We previously showed that Lamp1 and STARD3 co-localize in HeLa cells (see Figure EV2 in Wilhelm et al, EMBO J, 2017). The anti-Lamp1 antibody used here had little background and allowed to generate a robust late endosome/lysosome mask. Although we reached the same conclusion as before, because the quality of the images as well as the data were better with Lamp1 as an endosomal marker, they are now included in Fig. 6 and replace the previous data obtained with CD63. Please note that the relative filipin fluorescence in endosomes in the different cell lines is similar when using Lamp1 or CD63 staining. Besides, quantifications are now plotted using a Superplot (Lord et al, JCB, 2020) to show both individual cell values and the mean of independent experiments.

To verify the absence of fluorescence bleedthrough, we usually perform an experiment in which the various stainings are individually carried out (here filipin; anti-STARD3 AF488; anti-Lamp1 AF555), and images are then acquired in the different channels using the parameters (objective, laser intensity, time of acquisition, zoom…) used for the experiment where the different stainings are performed on the same sample (see Figure below). Here, fluorescence bleedthrough was really low, and considered negligible (compared to the pixel intensity usually observed in the different channels of a cell with the triple labeling).



Evaluation of fluorescence bleedthrough: stainings were individually carried out and images were acquired in the different channels using identical parameters. To evaluate fluorescence bleedthrough, we draw a mask comprising pixels with a high intensity (>150) and measured in the mask the fluorescence intensity in the other channels after background substraction. As shown on the right, fluorescence bleedthrough was really limited (<3%) and considered negligible.

o B, C: STARD3 and STARD3 S209A localization in C and in Fig4 D is different. Here it is more homogeneous, bigger aggregates. Is it due to the use of an inducible system? Can that affect the localization and filipin recruitment?

There are cell-to-cell variations regarding STARD3 localization, which is why we used an unbiased image analysis to quantify the data. The images in Fig 6 and 4 are illustrative of this cell-to-cell variability. However we agree with the reviewer and we checked the two expression systems, constitutive expression in Fig. 4, and Tet-inducible expression in Fig. 6. Pearson correlation analysis between STARD3 and Lamp1 in cells in which STARD3 expression was induced by doxycycline showed high correlation, with a Pearson correlation coefficient (PCC) of 0.7 (now shown on Fig. 6F) similar to that obtained with the constitutive system published in Wilhelm et al, EMBO J, 2017, Figure EV2 (STARD3/Lamp1 PCC

~0.75). Likewise, cholesterol accumulation visualized by filipin staining was similar using both expression systems (see Wilhelm et al, EMBO J, 2017 Figure 1 for the constitutive system and Fig. 6B this study).

Concerning the STARD3 S209A mutant, this question meets Reviewer 2 10th request. It is noteworthy that the expression of STARD3 S209A mutant affects the localization of late endosomes which become more perinuclear. This phenotype is not linked to a known role of STARD3 in late endosome trafficking, and we are currently investigating the potential role of STARD3 in late endosome positioning.

-The predictive algorithm suggests that 10% of the 20,000+ proteins they looked at have a phospho FFAT motif. This seems a very high hit rate for these algorithms and suggests that more stringent criteria for evaluation are necessary. Particularly, since it seems unlikely that all 2,000+ proteins will be able to by bind MSP domains.

We understand the point addressed here, the predictive position weight matrix is based on the primary sequence of proteins only. However, we have identified other criteria for the evaluation of a phospho-FFAT: i) to be exposed in the cytosol i.e. not inside an organelle, nor in a secreted protein ; ii) to be in an unstructured part of the protein i.e. not embedded in an alpha helix, a beta sheet, or a globular domain. To date, it is not possible to include these criteria in our analysis software because for most proteins, the subcellular localization, topology and 3D structure are not available. We are aware that this is a limitation of the analysis. We thought about crossing the data we obtained with data obtained with other prediction algorithms for the presence of unstructured regions in proteins, their localization in cells, and their topology if predicted to be inserted in a membrane. However, the gain would be mitigated by the increase in the number of false negative as predictive algorithms are not strongly reliable. We have chosen to include the results of the raw analysis with the position weight matrix in the manuscript.

To better explain this point, the ensemble of criteria necessary for a phospho-FFAT sequence to be effective are listed in the Results section:

"It is noteworthy that to be effective, a Phospho-FFAT sequence has to be in the cytosolic part of the protein, to be accessible to the MSP domain of VAP-A/VAP-B/MOSPD2, and is likely present in an unstructured region of the protein. Therefore, true Phospho-FFATs only represent a subset of this list."

Referee #2:

In this study, the authors investigate a mechanism by which membrane contact sites between the ER and other organelles could be regulated. The ER possesses three major tethering/receptor proteins (VAPA, VAPB, and MOSPD2) that are involved in the formation of contact sites with most

organelles. These three proteins contain an MSP domain allowing them to bind proteins with a motif named FFAT. The FFAT-motif containing proteins are diverse in their functions, and they include lipid transporters and ion transporters and apposition tethering proteins from almost all other organelles. Since these FFAT motif containing proteins grossly outnumber the ER tethering proteins, it suggests that interaction between the MSP domain and FFAT-motifs is likely tightly regulated. In this manuscript, the authors demonstrate how phosphorylation of the FFAT-motif may regulate binding with the MSP domain proteins. The authors identified proteins with a non-conventional FFAT motif with a serine or threonine at the 4th position of the motif. One such protein in the lipid transporter STARD3. Using various biochemical and cellular techniques, the authors demonstrate that the phosphorylation of the 4th serine residue is critical for STARD3 binding to the MSP domain proteins. Further, using both structural analysis and in vitro binding assays, the authors show a difference in the phospho-FFAT motif of STARD3 binding to MOSPD2 compared to binding to VAPA or VAPB.

This manuscript is a very robust study that provides a novel mechanism of the regulation of ER contact sites, which would be of great interest to a broad audience and particularly to the readers of EMBOJ. The authors used an impressive combination of a wide range of experimental techniques to establish the importance of the phosphorylation of the Phospho-FFAT motif of STARD3 in its interaction with VAPA/B and MOSPD2, as a prototype to study Phospho-FFAT motif and their regulation by phosphorylation. The finds presented in this manuscript add a new layer to our understanding of FFAT motifs and why they could be so variable. They also highlight differences between MSP domain-containing proteins (VAPs and MOSPD2). Moreover, structural and bio-informatics data provided with this study offers a new resource for the membrane contact site field. Overall, the manuscript is well written, and the authors are describing their experiments very clearly. The data are pretty convincing, and I have only some minor points that I think should be addressed.

Minor points:

• Fig 1C, Fig2C, and Fig7B: The negative control used should be stated in the figure legends (it is only in the main text).

We would like to thank the Reviewer for her/his insightful and positive comments on our manuscript.

The sequence identity of all the peptides used in the assay presented in Figure 1 including the negative control peptide sequence are now included in panel B. It is also the case for Figure EV3, peptides sequences including the control peptide are in panel A Fig. EV3. The text has been modified as follow: "These peptides, and a negative control peptide with a random sequence (Fig. 1B), were attached to streptavidin beads…".

The legends of Fig. 1, Fig. 2 and Fig. 7 were modified as follows:

Fig1B: "The negative control peptide is composed of a random sequence."

Fig. 2: “C: Recombinant MSP domains pulled down with peptides corresponding to the Phospho-FFAT motif of STARD3 (unphosphorylated; mono-phosphorylated on S_{209} or S_{213}; bi-phosphorylated on S_{209} and S_{213}), and with the control peptides (Fig. EV3 A) were revealed by Coomassie blue staining.”

Fig. 7: “B: Recombinant MSP domains pulled down with STARD3 FFAT peptides (unphosphorylated, mono-phosphorylated on S_{209} or S_{213}, bi-phosphorylated on S_{209} and S_{213}, or tri-phosphorylated on S_{203}, S_{209} and S_{213}.and control peptides (Fig. EV3 A) were revealed by Coomassie blue staining.”

• Fig 1E: the table should read "Phospho-FFAT" score and position and not "FFAT" score and position.

The table was modified as suggested (See Fig. 1F).

• Fig4: What are the FFAT (and/or Phospho-FFAT) score of the STARD3 mutants generated? Do the mutations increase or decrease this score? This should be stated in the legends and/or text.

There is a correlation between the scores and the introduction of either a disrupting mutation or a phospho-mimic mutation. The FFAT and Phospho-FFAT scores of the STARD3 mutants are shown below (color-coded as in Table S1 and S2):

	Phospho-FFAT	FFAT
STARD3 **WT**	2.5	6.5
STARD3 **S209A**	6,5	6.5
STARD3 **S209D**	6.5	2.5
STARD3 **S209D/P210A**	5.5	1.5

This is now indicated in the legend of figure 4:

“Please note that the mutations performed modify the FFAT and Phospho-FFAT scores of this region of STARD3: compared to WT STARD3 (Phospho-FFAT: 2.5; conventional FFAT: 6.5), the STARD3 S_{209}A, mutant has both low Phospho-FFAT and FFAT scores (6.5), the S_{209}D mutant has a high conventional FFAT score (2.5) but a low Phospho-FFAT score (6.5), and the double mutant a conventional FFAT score of 1.5 and a Phospho-FFAT score of 5.5.”

• Fig4: A STARD3 P210A construct with only the proline mutated in Alanine (without mutating the Serine) should be used to control that this mutation does not, by itself, promote the interaction with VAPA (or VAPB) either by Co-IP or confocal microscopy.

The structural analysis shows that the mutation P210A provides more flexibility to the peptide. This allows the sidechain of A_{210} to rest in the hydrophobic pocket formed by V51, T53, V61, N64 and

F95 (similarly to the side chain of A_{479} in ORP1) and to favor the interaction with VAP-A. Accordingly, by co-immunoprecipitation, we showed that STARD3 P210A interacted more pronouncedly with VAP-A than WT STARD3 (Fig. EV2D). We want to stress out that this mutation only increases the affinity of the Phospho-FFAT motif for the MSP domain and cannot induce an ON/OFF mechanism as the one observed with the phosphorylation on position 4. This is illustrated by the new other experiment added in Figure 1D which answers your last question. Indeed, in this experiment, we modified a perfect conventional FFAT motif (FFAT motif of ORP1) in synthetic peptides by replacing D478 by a serine, and by a phosphorylated serine. Interestingly, the FFAT motif of ORP1 possesses an alanine in position 5 (A479). In this context, the presence of an alanine in position 5 is not sufficient for the interaction: an Asp (WT ORP1) or a phosphorylated serine are absolutely required.

We added a new panel Fig. EV2 D and in the text:

"The single mutant STARD3 $P_{210}A$ showed an improved binding with VAP-A compared to WT STARD3 (Fig. EV2 D)."

• In Fig S6 (Fluorescence microscopy) or Fig 4C (Co-Ip), it seems that the STARD3 mutant S209D can interact with VAPB (although much less than WT or S209A/P210A) but not with VAPA (Fig4A and H). Can this small interaction be explained by structural differences in the MSP domain of VAPB compared to VAPA?

We did not observe a significant difference between VAP-A and VAP-B for the binding with the STARD3 mutant S209D: by co-IP, there is only a weak band (Fig. 4B) that is just above background; by fluorescence microscopy (former Fig. S6, now named Fig. EV4), the Pearson correlation coefficients between STARD3 S209D mutant and VAP-B are not statistically different from the ones between STARD3 S209A mutant and VAP-B, which shows that the S209D mutation behaves as a non-binder or a really weak binder.

To directly investigate this possibility, in this revised version of the manuscript, we added peptide pull-down experiments performed with peptides possessing the S209D and the S209D/P210A mutation, and recombinant proteins (VAP-A, VAP-B and MOSPD2) (now shown Fig. EV2 B). The peptide with the S209D mutation did not pull-down VAP-B (or VAPA).

Moreover, we did not observe structural differences in the MSP domain of VAP-A and VAP-B in the FFAT/Phospho-FFAT interaction surface.

• Fig5: In the main text the authors conclude that "Thus, in a complete in vitro defined system, the sterol transport function of STARD3 is turned on by phosphorylation of its FFAT motif". In other words, the authors suggest that the phosphorylation of its FFAT motif directly regulates the lipid transfer ability of STARD3. However, this is an over-interpretation of their data. Taken together with their binding data, more likely interpretation of the liposome data is that S209 phosphorylation in

STARD3 is required to establishment contact between the liposome via its being with VAP. And this close contact between these liposomes allows STARD3 to transfer DHE between them. To demonstrate whether this phosphorylation directly activates the sterol transfer ability of STARD3 the authors should test the DHE transfer in the presence of artificial tethers that would mediate the contact of liposomes between them independently of STARD3-VAP and with the FFAT domain of STARD3 phosphorylated or not (and with or without VAP). Otherwise, the authors should modify this statement in the main text.

We would like to thank the reviewer for pointing out this poorly phrased sentence. We totally agree with her/his interpretation, and therefore corrected this sentence as follows:

"Thus, in a complete *in vitro* defined system, phosphorylation of STARD3's Phospho-FFAT drives membrane tethering by enabling STARD3/VAP complex formation that in turn allows the efficient transport of sterols mediated by the START domain."

• Fig. 5D: In the DHE transport experiment, the authors hypothesize that the decrease in the FRET signal is due to the transfer of DHE from LB to LA by pS209 cSTD3. However, similar results can occur if the two liposomes fuse. The authors need to rule out that fusion is not occurring between the liposomes.

To test if liposome fusion happens during the DHE transport experiment, we performed a FRET assay which is shown in a new figure (Fig. EV5). This experiment, based on a standard FRET assay using an NBD-PE/Rhod-PE pair, showed that no liposome fusion occurs when liposome attachment was induced by the interaction of pS209 cSTD3 with VAP-A.

The assay is described in the Materials and Methods section (Fusion assay paragraph), and the results are indicated in the text:

"It is noteworthy that the attachment of membranes does not provoke any fusion (Figure EV5) as measured by a standard FRET assay using an NBD-PE/Rhod-PE pair (Struck et al, 1981)."

• Fig6: Does the overexpression of the phosphomimetic S209D/P210A promotes the accumulation of cholesterol in late endosomes? According to Fig4E, this mutant can interact with VAPA to establish ER endosome contact site. Therefore, the authors should include the S209D/P210A mutant in this assay to examine whether it acts as a constitutive active motif similar to the Conv-FFAT.

As recommended by the reviewer, cholesterol accumulation in the presence of the phosphomimetic STARD3 $S_{209}D/P_{210}A$ mutant was measured using a filipin staining assay. The results are shown in the new version of Figure 6. The expression of STARD3 $S_{209}D/P_{210}A$ mutant induced cholesterol accumulation in late endosomes similarly to WT STARD3 and STARD3 with a conv-FFAT.

• Fig 4 A, H and Fig7 F: It seems that the phosphomimetic mutants (S209D/P210A for VAPA and S209D for MOSPD2) do not interact as strongly as the wild-type STARD3 as evaluated by co-IP or

colocalization. It is counter-intuitive as the phosphomimetic mutations should block STARD3 in an "ON" state for the interaction with VAPA or MOSPD2. Could the authors comment on that? Can this be explained by structural data showing the limitations of phosphomimetic mutations in this case?

Indeed, the phosphomimetic mutants interact slightly less than wild type STARD3. There are structural differences that might explain this observation: the phosphoserine pS_{209} makes interactions with K50 and K52 of VAP, and K363 and R365 of MOSPD2. Replacing S_{209} by an aspartate is less favorable for the interactions: the acidic residue is shorter and have less possibilities to establish contacts (2 oxygen atoms vs 3, thus charge densities are different). For VAP, the replacement of S_{209} by an Asp is therefore not sufficient to restore the interaction. For MOSPD2, the Arg residue (R365), has more interacting sites than lysine, and can therefore accommodate to recognize the S209D mutation, restoring binding and affinity. Moreover, the Phospho-FFAT of STARD3 comprises 2 prolines which impose geometrical constraints. Even if the $S_{209}D/P_{210}A$ mutation allows the binding with VAP, there is still one remaining proline in the motif. The lack of flexibility imposed by prolines might also explain why a phosphomimetic is not equivalent to a phosphoserine.

• Fig 4D, 6C and S6C: the overexpression of STARD3 S209A appear to promote the re-localization of Late endosomes to the perinuclear region. Is this true? Is it linked to a known role of STARD3 in late endosomes trafficking?

As noted by this reviewer, the expression of the STARD3 S209A mutant affects the localization of late endosomes which become more perinuclear. This phenotype is not linked to a known role of STARD3 in late endosome trafficking, and we are currently investigating the potential role of STARD3 in late endosome positioning.

• In a perfect conventional FFAT motif (FFAT score = 0), if the Asp at the fourth position is replaced by a Ser is there still an interaction with MSP domain, or does it require the phosphorylation of the Serine (for example in a pull-down assay as Fig1C or 2C)?

To answer this question, we used the perfect conventional FFAT motif of ORP1 (Score=0). As it was originally shown in Figure EV2C, this motif binds VAP-A, VAP-B and MOSPD2 in peptide pull-down experiments. The acidic residue in position 4 of ORP1's FFAT motif was modified in synthetic peptides by replacement of D478 by a serine, and by a phosphorylated serine. We then performed peptide pull-down experiments as in Fig EV2C. The results are shown in Figure 1D. The mutant peptide bearing a D478S replacement did not pull-down VAP-A, VAP-B or MOSPD2, while the peptide bearing a phosphorylated serine (D478pS) pulled down VAP-A, VAP-B and MOSPD2.

These results are included in Fig.1D and are described in the text.

Referee #3:

This study by Di Mattia et al. identifies and characterizes special type of FFAT motifs in the human proteome that are regulated by phosphorylation. Conventional FFAT (two phenylalanines in an acidic track) motifs provide interaction of proteins with the ER localized VAP-A and VAP-B proteins as well as with the recently described MOSPD2 protein at ER-to other organelle membrane contact sites. This study used an in-silico approach to identify FFAT motifs in which phosphorylatable serine or threonine residues substitute for the conserved D/E residues in position 4 of conventional FFAT domains. This analysis identified 110 proteins that scored high with these analyses. Two of these proteins, Kv2 potassium channels have already been described with such a phospho-regulated FFAT-like motif.

The authors then selected one of the proteins, STARD3 for an extensive analysis of its FFAT-like domain using an impressive repertoire of assays. These included peptide binding to recombinant MSP domains (the domains within the VAP and MOSPD2 proteins that interact with FFAT motifs) and determination of the X-ray structures of the human VAP-A and MOSPD2 MSP domains with the bound STARD3 FFAT peptide. Moreover, the Authors analyzed the role of the phospho-FFAT motif of STARD3 in its interaction with the VAP and MOPSD2 proteins in intact cells, and the role of this interaction in cholesterol transport both analyzed in intact cells and in an in vitro liposome transfer assay. Collectively, these studies convincingly showed that phosphorylation of the key serine residue (S209) in STARD3 is critical for its function as a sterol transfer protein between the ER and endosomes.

The topic of non-vesicular lipid transport between the ER and other organelles has received enormous attention in recent years because of its importance in cellular lipid metabolism and membrane biology with ample connection to human diseases. FFAT motifs found in many lipid transfer proteins have been well documented as a critical structural element for the proper function of these proteins. The discovery that about equal number of phospho-regulated FFAT motifs exist in the human genome is a very important step in this field that will allow future studies to explore their importance in the many proteins identified in this study.

The experiments featured in this work are very well-done and well-documented, convincingly supporting the conclusions. If anything, the Authors present more data and supplementary data than would be suffcient to make their case. However, all of these experiments are strong and well-organized and should be kept for the interested reader.

I have very few comments that might be useful to address to make the paper more accessible for the average reader.

1. The authors should describe better the various screening algorithms. For example, it is not clear what the score 1 and score 2 means in Tables S1 and S2?

We want to thank the Reviewer for her/his helpful comments on our manuscript.

To facilitate the comprehension of the algorithm, an editable Microsoft Excel file allowing users to calculate the Phospho-FFAT score of a given protein is included as a Supplementary table (Table S4). This file is composed of two sheets: 1 sheet that describes the algorithm itself (with the different weights of residues in the matrix), and the second sheet in which the user can paste the sequence of a protein (C2 cell labeled "Protein"), indicate the position of the first residue of the sequence to be analyzed (C3 cell labeled "start"); the excel file then calculates the different sub-scores and the final score for this specific sequence.

The algorithm calculates a score at each position of a given protein (n scores calculated for a protein of n residues). Scores 1 and Score 2 are selected as the two best scores in a given protein. This is now indicated in the materials and methods section

"Thus, for proteins with n residues, n scores were calculated; the two best scores (named SCORE1 and SCORE2) are shown Table S1 and S2."

2. Please, define DHE (dehydroergosterol) and DNS-PE (dansyl)-PE in the text and in Figure legends (Fig. 5)

DHE and DNS-PE are now defined in the text and figure legend.

"To do so, we measured the intermembrane transfer of the fluorescent sterol dehydroergosterol (DHE) in real time by FRET (Fig. 5D). L_B liposomes including both DHE (10 mol%) and a second fluorescent lipid, dansyl-phosphatidylethanolamine (DNS-PE,2.5 mol%), were covered with VAP-A_{-His6} and added to L_A liposomes decorated with pS_{209} cSTD3 (Fig. 5B). The transport of DHE from L_B to L_A liposomes was followed by measuring the decrease in energy transfer from DHE to DNS-PE. A fast transport of DHE was observed within the first seconds and DHE was entirely equilibrated between the two liposome populations after a few minutes."

Figure 5 legend:

"For dehydroergosterol (DHE) transport experiment, L_B liposomes also contain DHE and a dansylated phosphatidylethanolamine (DNS-PE)."

3. Also, please describe the principle of the liposome assay (FRET) and what the curves mean in Fig 5D.

The liposome assay principle is better described in the results section:

"To do so, we measured the transfer of the fluorescent sterol dehydroergosterol (DHE) in real time by FRET (Fig. 5D). At the beginning of this assay, DHE fluorescence is quenched by the FRET acceptor dansyl-phosphatidylethanolamine (DNS-PE). The transport of DHE from LB to LA liposomes is followed

by the decrease in FRET between DHE and DNS-PE. L_B liposomes including both DHE (10 mol%) and DNS-PE (2.5 mol%), and covered with VAP-A_{-His6} were added to L_A liposomes decorated with pS_{209} cSTD3 (Fig. 5B)."

The meaning of the curves Fig. 5D is now explained in the legend:

"FRET between DHE and DNS-PE in the LB liposomes diminishes as DHE is transported towards LA liposomes. The signal was converted into amount of DHE present in LB liposomes (in mol%)."

4. Was the binding of phosphomimetic pS209 peptides to VAP-MSP domains tested in the recombinant assays? Convincingly it was not sufficient in the cellular assays using the full-length protein.

The phosphomimetic mutant of the Phospho-FFAT (S209D and S209D/P210A mutants) were tested in in vitro assays using recombinant proteins. The results are shown Fig. EV2 B and described in the text:

"In agreement with these data, the recombinant MSP domain of VAP-A and VAP-B interacted with peptides corresponding to the STARD3 $S_{209}D/P_{210}A$ mutant motif, while it did not interact with peptides having the STARD3 $S_{209}D$ mutant motif (Fig. EV2 B)."

The mutants behaved similarly in in vitro and in cellular assays.

5. What are the predictions for binding of the phosphor-FFAT of STARD3 to the ALS mutant MSP domain of VAP-B(P56S)? Was it tested by any chance?

The biochemical properties of the VAP-B P56S mutant have been extensively characterized by the Sima Lev's laboratory (Kim et al, JBC, 2010). They have shown that the P56S mutation facilitates the exposition of hydrophobic patches of the protein to the solvent, and VAP-B P56S is therefore prone to aggregation. However, VAP-B P56S retains its ability to bind the FFAT motif. When expressed in cells, the VAP-B P56S mutant forms large cytoplasmic aggregates which exclude FFAT-containing proteins. Due to the insolubility of VAP-B P56S, we did not perform *in vitro* experiments to test its ability to bind Phospho-FFATs. Based on the work of the Lev's lab, this mutant should be able to bind Phospho-FFATs in principle, but as the protein aggregates it should not recruit Phospho-FFAT containing proteins.

6. Were there other serines phosphorylated in the phospho-cSTD3 prep from E.Coli or only P209?

The production of the phospho-cSTD3 recombinant protein was performed in the E. coli C321 ΔA strain in which all natural Amber stop codons (UAG) were removed (Lajoie MJ et al, Science 2013). This strain was transformed with an orthogonal translation system (Pirman et al, Nat Commun. 2015) using

the Amber codon to allow the site-specific incorporation of Phospho-serine. To this aim, the plasmid encoding cSTD3 (STARD3 [196–445]) was modified by site-directed mutagenesis to replace S_{209} codon by an amber codon (TAG). This system is thus designed to incorporate phospho-serine at only one given position in the recombinant protein. Accordingly, the mass spectrometry analysis (Figure Appendix S3) showed that: i) the recombinant protein was present in two forms regarding phosphorylation, one without phosphorylation and one with a single phosphorylation (Figure Appendix S3 A); ii) while the protein contains 20 serines distributed along the primary sequence, after trypsin digestion, only the peptide comprising S209 displayed a phosphorylation (Figure Appendix S3 B and data not shown); iii) the MS/MS analysis unambiguously showed the presence of phosphorylation on S209, and did not detect phosphorylation on any other serine (Figure Appendix S3 B and data not shown). Together, these data show that S209 was the sole phosphorylated serine present on phospho-cSTD3 recombinant protein.

7. Does the MOSPD2 MSP domain pFFAT of STARD3 peptide interaction in the X-ray structure explain why the phosphomimetic mutant binds here but not in the VAP-A MSP domain?

There are two aspects that explain why the STARD3 S209D mutant binds MOSPD2 and not VAP-A. First, in the structure, the phospho-serine interacts with an arginine and a lysine in MOSPD2, while it interacts with 2 lysines in VAPA; the arginine (R365) in MOSPD2, unlike lysines, probably restores the interaction (see above, Referee #2 Q/A 9). Second, compared to VAP, there is an additional hydrophobic pocket in MOSPD2 that accommodates F214, and which strengthens the interaction.

8. Table S2 has 110 (line 18 on page 5) or 109 (line 29, page 5) partners. Should not they be the same?

Table S2 (now Table EV2) has 110 partners. This was corrected in the text.

2nd Revision - Editorial Decision 25th Aug 2020

Thank you for submitting your revised manuscript. The study has been seen by the original referees, whose comments are shown below.

As you can see, the reviewers find that their criticisms have been sufficiently addressed and recommend the manuscript for publication. However, there are a few editorial issues concerning the text and the figures that I need you to address before we can officially accept your manuscript.

Referee #1:

The authors have addressed all my comments with further experiments or with clarifications in the text:
- They showed that the localization of STARD3 mutants in a STARD3 silenced background is the same as in the overexpression experiments, and they analyzed VAP/STARD3 colocalization by Pearson correlation in silenced STARD3 cells. This proves that endogenous STARD3 is not competing for the VAP binding site.
- Figure 6 was updated with a better endosome/lysosome marker, LAMP1, and a bleedthrough analysis was performed. Images are now clearer, and staining is more specific.
-The cell-to-cell localization differences for STARD3 was addressed by Pearson correlation to show that it doesn't affect flipin recruitment and that the results are similar for both expression systems.
The modifications have improved the manuscript and I endorse publication in the EMBO Journal.

Referee #2:

The authors have addressed all of my concerns and I support the publication of this article.

Referee #3:

This is a revised version of the manuscript by Di Mattia et al.,in which the Authors identify and characterize special type of FFAT motifs in the human proteome that are regulated by phosphorylation. Conventional FFAT (two phenylalanines in an acidic track) motifs provide interaction of proteins with the ER localized VAP-A and VAP-B proteins as well as with the recently described MOSPD2 protein at ER-to other organelle membrane contact sites.
As I pointed out in my original review, this study is important as it identifies phospho-regulation of special FFAT-motifs which can control organelle contact sites and lipid transfer processes. The study is technically very solid and the data convincingly support the conclusions.
The Authors have addressed my original concerns, which improved the clarity and accessibility of the manuscript for the general readership.
I consider this work now acceptable for publication.

2nd Authors' Response to Reviewers — 2nd Sep 2020

Please find a modified version of our revised manuscript entitled “Phosphorylation switches on inter-organelle contacts“ (EMBOJ-2019-104369R1).

3rd Revision - Editorial Decision — 7th Sep 2020

I am pleased to inform you that your manuscript has been accepted for publication in The EMBO Journal.

Congratulations!

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: ALPY Fabien
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2020-105120

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 iustified
- ➔ 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.

USEFUL LINKS FOR COMPLETING THIS FORM

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

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-tumo

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://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size for the studies was chosen according to previous studies in the same area of research. In general 3 to 6 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.	The data were analyzed using non-parametric tests (Mann-Whitney or Kruskal-Wallis)
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.

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

D- Animal Models

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

E- Human Subjects

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

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Protein structures were deposited in the PDB database. We added a "Data availibility" section in the Materials & Methods section.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	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