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Review COMMONS

The Hob proteins are novel and conserved lipid-binding proteins at ER-PM contact sites

Sarah D. Neuman, Jeff R. Jorgensen, Amy T. Cavanagh, Jeremy T. Smyth, Jane E. Selegue, Scott D. Emr and Arash Bashirullah
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Reviewer 1

Evidence, reproducibility and clarity

This study shows that the Drosophila protein Hobbit and its yeast homologue Fmp27 localize to contact sites between the ER and plasma membrane. Both proteins are shown to bind the ER via their N-termini and the plasma membrane by their C-termini. The C-terminal domain from Hobbit binds phosphoinositides immobilized on a membrane. Consistent with previous work from this group, flies lacking Hobbit have small pupa and this study shows both the N- and C-terminal regions are necessary for the protein to function. The study concludes that Hobbit and Fmp27 may play a role in lipid trafficking and intracellular PI(4,5)P2 distribution.

The study is well done and well presented. However, the idea that Hobbit and Fmp27 affect lipid trafficking, while reasonable, is not supported by any direct evidence. The finding that PI(4,5)P2 distribution is altered in salivary gland cells does not, by itself, indicate that Hobbit affects lipid transport or even directly affects phosphoinositide metabolism.

Significance

The primary advance in this study is that it identifies a new family of proteins at ER-plasma membrane contact sites and binds to phosphoinositides. While this is certainly interesting, a number of other families of proteins with similar properties have already been identified. For this study to be appropriate for EMBO J or a similar journal, more insight into the function of Hobbit and Fmp27 is necessary. Without that, I think this is more appropriate for more specialized journals like MBoC, JCS, or EMBO Reports.

Reviewer 2

Evidence, reproducibility and clarity

Here Neuman et al. investigate the yeast and Drosophila hob proteins. They conclude that these proteins are localized to ER-PM contact sites in both experimental systems. However, yeast Fmp27 does not appear to be an ER-PM tether. The developmental defects observed in Drosophila hobbit mutants point to an important functional role of these proteins. Although these findings are interesting, I have a number of concerns about the quality and interpretation of the data presented here.

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Major comments

- Figures: many micrographs don't have scale bars (e.g. Fig 1, Fig 2, Fig 3). Others have minute, barely visible bars (e.g. Fig 4).

- Data presentation: most of the findings presented here are not quantified, e.g. all light microscopy and biochemistry. The few quantifications shown (Fig. 3B, Fig. 6C) are not statistically analyzed. There is also no indication of how many times the experiments were repeated. Please include this information. Please quantify the data and describe only differences that are statistically significant.

- Fig 1, Fig S1: Compared to Tcb3, Fmp27-GFP does not localize only to the cER, there is also signal in the rest of the ER including the nuclear envelope. This needs to be acknowledged throughout the manuscript, e.g. in sentences like "providing further evidence that Fmp27 localizes to ER-PM contact sites". A quantification of the colocalization of Fmp27-GFP with RFP-HDEL and Tcb3-mCherry would be very useful.

- Bioinformatic structural predictions should be shown. In the light of Fig. S1C, it is not clear why it was decided to truncate the protein at AA192. The first 192 AAs are not "highly conserved" as the authors write, but rather less conserved than the rest of the protein. The truncated protein seems to retain certain cER localization (Fig 2A), suggesting that the truncation site may not be optimal. Also, the proteinase K experiments only inform about the location of the N-terminus, but not about the number of transmembrane segments, so sentences like "These results demonstrate that Fmp27 is anchored to the ER via an N- terminal transmembrane domain or hairpin" or "The topology of Fmp27, with an N-terminal membrane anchor and a large cytosolic domain" don't seem to be justified. I also do not understand the Kar2 control: the antibody seems very dirty and a huge number of bands are present.

- Please show (at least some of) the EM images used for Fig 3B.

- Fig 4A: I am not familiar with Drosophila salivary glands, but I do not see how Fig. 4A supports ER localization of hobbit. Hob-GFP clearly does not colocalize with KDEL-RFP, and at this resolution it would certainly not be possible to distinguish proteins localized in the lumen or the membrane of the ER.

- Fig 4B: Was a secondary structure prediction run for Drosophila hobbit? How similar is it to Fmp27? Although in this case it is more clear how the truncation site was chosen (Fig S2B), for me it is not obvious where the protein is localized in Fig 4B. It still follows some kind of pattern, not fully colocalizing with mTagBFP2. Why is mTagBFP2 so different in Fig 4A and Fig 4B? Is ER morphology affected by expression of Hob?N117-GFP? (KDEL-RFP puncta look smaller in Fig 4B than in Fig 4A). "Importantly, ER localization is critical for hobbit function, as ubiquitous overexpression of hob?N117-GFP did not rescue the small body size or lethality of hobbit mutant animals": how do know this truncation does not have other effects beyond (presumably) affecting protein localization?

- The same applies to Fig S3B: how can we conclude from these images that Hobbit?C-GFP localizes to the ER? Also, it is unclear how the truncation site for Fmp27 was chosen (Fig S4A).

- Fig 4C suggests that the protein is inserted in some intracellular membrane, not necessarily ER.

- Fig 5: again, I am not familiar with this system, but how do we know those puncta are at the PM? "Additionally, StimDDAA-GFP appeared to localize normally in hobbit mutant cells (Fig. 5A)": those mutants have not yet explained in the text nor in the figure legend. Altogether, the ER/ER-PM localization of hobbit needs to be demonstrated more clearly.

- Please show the Pfam predictions, as well as the sequence comparisons with Atg2 and Vps13.

- It would be important to test binding of Apt1 to other PIs: is there selectivity for any specific PI? The text says "These lipid moieties are known to be enriched at the plasma membrane", but PIs are present at many other membranes. Without understanding if there is any PI specificity, we cannot

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conclude that "ER-PM localization of Hobbit may be mediated by binding to plasma membrane phosphatidylinositols". "Fly Hobbit Apt1 bound to PI, PI(4)P, PI(4,5)P2, and PI(3,4,5)P3, while yeast Vps13 bound to PI(3)P and human VPS13A bound to PI(3)P and PI(5)P (Kolakowski et al., 2020; Rzepnikowska et al., 2017). Thus, Apt1 domains may represent a novel family of phosphatidylinositol binding domains with varying specificity": how can this comparison be established if binding to PI(3)P and PI(5)P was not tested for hobbit?

- "However, our data presented here suggests that the molecular function of hobbit lies in the regulation of phosphatidylinositol homeostasis": I do not think the data shown in Fig 7B suffice to establish the main function of hobbit.

Minor comments

Summary
- "Higher animals" should be substituted by "Drosophila". Introduction
- "ER-PM contact sites are particularly ubiquitous and prevalent in yeast": define "yeast" as "S. cerevisiae". There are of course other yeast species, but the following text refers only to S. cerevisiae.
"However, surprisingly, ER-PM contact sites are not required for yeast viability under normal laboratory growth conditions": it's worth mentioning that ER-PM contact sites are important under stress conditions such as heat shock (e.g. PMID 26864629, 31743662).

- Conservation: to what extent are Hobbit proteins conserved in vertebrates? Results
- Perhaps it would make sense to rename Fmp27, as "Found in Mitochondrial Proteome" doesn't seem appropriate in the light of this manuscript.
- Fig 6 is mentioned in the text before Fig 5.

Significance

In principle the findings reported here will be of interest to a broad cell biology audience. However, these findings need to be substantiated better before final publication, as described above. It would also be interesting to describe in more detail the conservation of Hobbit proteins in vertebrates, and the possible roles they may play there. My expertise: structure of membrane contact sites.

Reviewer 3

Evidence, reproducibility and clarity

The authors present a straightforward series of experiments (done in yeast and flies) that characterizes Hobbit proteins as occupants of ER-PM contact sites. The data are of good quality and the conclusions are clear: yeast and fly hobbit are ER-anchored proteins with a lumenal N-terminus and extensive C-terminus, the last portion of which comprises a phosphoinositide-binding Apt1 domain that associates the protein with the PM and is needed for function. Of interest, without the Apt1 domain, the localization of phosphoinositides at the PM is dysregulated. I have some minor points for the authors to attend to, but also a significant comment about their unsupported claims that Hobbit could play a role in intermembrane lipid transfer.

Page 3, three lines from the end of the second paragraph, & page 4, 5 lines from the end of the top paragraph: there is no evidence that ER-PM contact sites play a role in sterol transfer/trafficking in yeast. In fact, the opinion article by Dittman/Menon (TIBS 2017) and the experimental test of this claim by Quon et al. (2018) indicate the opposite.

Page 5, last sentence of Introduction: there is no evidence to support the claim that Hobbit may be a lipid transfer protein

Page 8, Fig 3B: representative images should be shown, at least in the supplement

Page 10, Fig 4A and 4B: enlarged views (such as shown in Figure 7) are needed as it is

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impossible to clearly see the described morphology. This could be done by providing insets.

Fig 4C: it is surprising that the digitonin permeabilization method works well in drosophila cells given that they may not be as sterol-rich as mammalian cells. The authors may want to comment on this.

Fig S4: the expression level of the deletion mutant is much less than that of the full-length protein. A comment would be appropriate.

Page 14: While reading I was at a loss to know what the Apt1 domain was. This becomes clear in the discussion section. A short explanation would be useful in the resuts, with a parentetical statement such as 'see discussion for more detail'.

Discussion, last sentence first para: there is no evidence that Hobbit is a lipid trafficking regulator. The authors have shown that it affects the distribution of steady state pools of phosphoinositide. This could be the result of many factors.

Discussion, page 16, para 3: This sentence is also not justified - there are no data to support it (see comment above). "Given that Hobbit binds to phosphatidylinositols and that PI(4,5)P2 distribution appears to be altered in hobbit mutant cells, our data suggests that hobbit may be a novel lipid transfer protein at ER-PM contact sites"

Discussion, page 17, top para, last sentence: again not justified - see above.

Significance

This paper describes interesting new results on a new protein at ER-PM contact sites. The protein appears to localize to these sites without contributing a tethering function, and plays a role in regulating the distribution of phosphoinositides between the PM and an intercellular compartment. The authors over-reach by deducing that Hobbit is a lipid transfer protein - they have no data to support this claim. While undoubtedly interesting, the data represent just the first steps in looking into these proteins and therefore seem a little preliminary. Consequently, the paper could just as easily be published in a number of other journals, for example Mol Biol of the Cell or J Biol Chem.

Author response to reviewers' comments

We sincerely thank the reviewers for their thoughtful and constructive comments. In response, we have added several new figure panels, as well as additional quantification and statistical analysis to our manuscript. We have also made significant edits to the manuscript text. As a result, we feel that this is a much stronger and clearer story. Thank you!

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

This study shows that the Drosophila protein Hobbit and its yeast homologue Fmp27 localize to contact sites between the ER and plasma membrane. Both proteins are shown to bind the ER via their N-termini and the plasma membrane by their C-termini. The C-terminal domain from Hobbit binds phosphoinositides immobilized on a membrane. Consistent with previous work from this group, flies lacking Hobbit have small pupa and this study shows both the N- and C-terminal regions are necessary for the protein to function. The study concludes that Hobbit and Fmp27 may play a role in lipid trafficking and intracellular PI(4,5)P2 distribution.

The study is well done and well presented. However, the idea that Hobbit and Fmp27 affect lipid trafficking, while reasonable, is not supported by any direct evidence. The finding that PI(4,5)P2 distribution is altered in salivary gland cells does not, by itself, indicate that Hobbit affects lipid transport or even directly affects phosphoinositide metabolism.

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We thank the reviewer for these supportive and constructive comments. We have edited the text of our manuscript to make it clear that a possible lipid transfer function for the Hob proteins is speculative, and that additional work will be required to directly test this hypothesis.

Reviewer #1 (Significance (Required)):

The primary advance in this study is that it identifies a new family of proteins at ER-plasma membrane contact sites and binds to phosphoinositides. While this is certainly interesting, a number of other families of proteins with similar properties have already been identified. For this study to be appropriate for EMBO J or a similar journal, more insight into the function of Hobbit and Fmp27 is necessary. Without that, I think this is more appropriate for more specialized journals like MBoC, JCS, or EMBO Reports.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Here Neuman et al. investigate the yeast and Drosophila hob proteins. They conclude that these proteins are localized to ER-PM contact sites in both experimental systems. However, yeast Fmp27 does not appear to be an ER-PM tether. The developmental defects observed in Drosophila hobbit mutants point to an important functional role of these proteins. Although these findings are interesting, I have a number of concerns about the quality and interpretation of the data presented here.

Major comments

- Figures: many micrographs don't have scale bars (e.g. Fig 1, Fig 2, Fig 3). Others have minute, barely visible bars (e.g. Fig 4).

We have now added scale bars to all micrographs throughout the manuscript and have increased the size and thickness of the bars to make them easier to see.

- Data presentation: most of the findings presented here are not quantified, e.g. all light microscopy and biochemistry. The few quantifications shown (Fig. 3B, Fig. 6C) are not statistically analyzed. There is also no indication of how many times the experiments were repeated. Please include this information. Please quantify the data and describe only differences that are statistically significant.

We have added additional quantification and statistical analysis to our revised manuscript. We have also included reproducibility details in each of the figure legends.

- Fig 1, Fig S1: Compared to Tcb3, Fmp27-GFP does not localize only to the cER, there is also signal in the rest of the ER including the nuclear envelope. This needs to be acknowledged throughout the manuscript, e.g. in sentences like "providing further evidence that Fmp27 localizes to ER-PM contact sites". A quantification of the colocalization of Fmp27-GFP with RFP-HDEL and Tcb3-mCherry would be very useful.

We have edited the Results text to state that Fmp27 is present throughout the ER but enriched at ER-PM contact sites. Fmp27 is expressed at quite low levels endogenously; thus, the amount of background/autofluorescence makes quantification of co-localization difficult. Instead, we have quantified co-localization of fly Hobbit with constitutively active Stim (new Fig. 5D) and find that these two proteins strongly co-localize. Furthermore, there is a significant reduction in co-localization between $Stim^{DDAA}$ and HobΔC82 (new Fig. 5D).

- Bioinformatic structural predictions should be shown. In the light of Fig. S1C, it is not clear why it was decided to truncate the protein at AA192. The first 192 AAs are not "highly conserved" as the authors write, but rather less conserved than the rest of the protein. The truncated protein seems to retain certain cER localization (Fig 2A), suggesting that the truncation site may not be optimal. Also, the proteinase K experiments only inform about the location of the N-terminus, but not about the number of transmembrane segments, so sentences like "These results demonstrate that Fmp27 is anchored to the ER via an N-terminal transmembrane domain or hairpin" or "The topology of

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Fmp27, with an N-terminal membrane anchor and a large cytosolic domain" don't seem to be justified. I also do not understand the Kar2 control: the antibody seems very dirty and a huge number of bands are present.

We apologize that we didn't make the intent of our sequence analysis studies clear in the initial version of the manuscript. The Hob proteins have not been structurally characterized; thus, to make an informed decision about where to truncate the protein for our initial studies, we relied on primary sequence analysis. Our intent was to identify 'blocks' of conserved sequences separated by 'gaps' with minimal sequence conservation, truncating the protein at the junction of these 'blocks'. We have edited the Results and figure legend text to clarify this point. Additionally, it is clear that N-terminally truncated Fmp27 no longer localizes to the ER; however, this truncated protein does still contain the C-terminal sequences, including the Apt1 domain, that bind to lipids and are required for cER enrichment. Thus, the few cER puncta in Fig. 2A may be a result of low-level binding to plasma membrane lipids by these C- terminal sequences.

The Kyte-Doolittle hydrophobicity analysis (Fig. S2A) shows a single, highly hydrophobic region at the N- terminus of Hobbit (the first 18 amino acid residues), a profile consistent with a single membrane anchor. Additionally, the fact that ER localization is lost in both Fmp27ΔN192 and HobbitΔN117 suggests that there are no additional transmembrane domains within the Hobbit protein. Nevertheless, it is formally possible that there are additional undetected transmembrane domains within Fmp27/Hobbit, and we have softened our statements to reflect this.

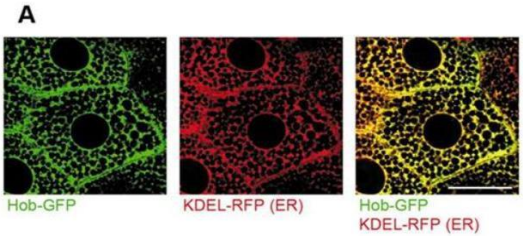
Finally, the Kar2 antibody was initially generated by the Rose lab (Rose et al 1989) and later re-generated using the same antigen by the Schekman lab (Brodsky & Schekman 1993). This antibody has since been used in a number of publications (e.g. Mullins et al 1995; Baxter et al 1996; Robinson et al 1996), including for protease protection assays (Shen et al 2009). Since this is not a commercial antibody, there are some non-specific bands present. However, in the interest of full transparency, we have chosen to show the full, uncropped blot with these non-specific bands in the main figure instead of including it as supplemental data.

- Please show (at least some of) the EM images used for Fig 3B.

We have added a representative image in Fig. S1D.

- Fig 4A: I am not familiar with Drosophila salivary glands, but I do not see how Fig. 4A supports ER localization of hobbit. Hob-GFP clearly does not colocalize with KDEL-RFP, and at this resolution it would certainly not be possible to distinguish proteins localized in the lumen or the membrane of the ER.

The *Drosophila* larval salivary glands are composed of massive cells that are about 100 µm in diameter; thus, we are able to image subcellular structures at very high resolution. Our previously published work shows that full-length Hobbit-GFP co-localizes with KDEL-RFP when imaged at lower magnification/resolution (Neuman & Bashirullah 2018). The image below from this paper illustrates this point. This image was taken in salivary glands prior to the onset of developmentally programmed mucin secretion; the "black" areas of the images outline these granules (the large black area in the center of each cell is the nucleus).



Full-length Hobbit-GFP and KDEL-RFP still co-localize in salivary glands after the completion of mucin secretion (at the onset of metamorphosis; 0 h PF); however, Hobbit-GFP and cytosolic mCherry do not co- localize, nor do KDEL-RFP and cytosolic GFP, as shown below (Neuman & Bashirullah 2018).

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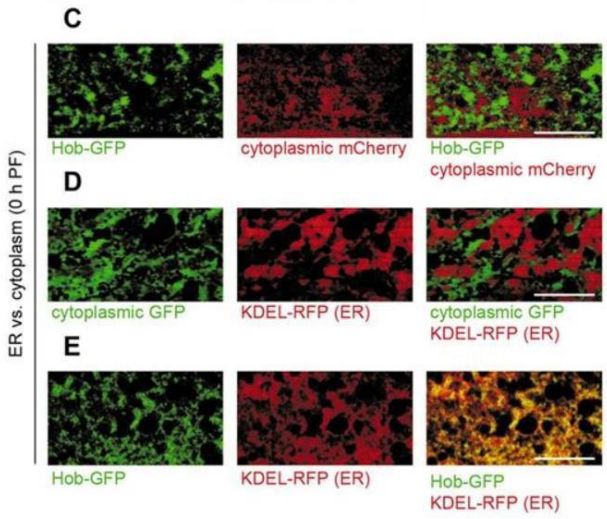

In this manuscript, we have extended this analysis to image Hobbit localization at much higher magnification and resolution. The images in Fig. 4 A-B and Fig. S3B were acquired at a resolution of 77 nm/pixel. Additionally, the ER appears to undergo a rapid, developmentally programmed remodeling after the conclusion of mucin secretion. This works to our advantage, because one stage of this remodeling process appears to involve temporary fragmentation of the ER, with the sheets and tubules “rounding up” to form the “balls” or puncta seen in Figs. 4A-B and S3B. This gives us an even greater spatial capacity to differentiate between the ER lumen and the ER membrane with super resolution imaging.

- Fig 4B: Was a secondary structure prediction run for Drosophila hobbit? How similar is it to Fmp27? Although in this case it is more clear how the truncation site was chosen (Fig S2B), for me it is not obvious where the protein is localized in Fig 4B. It still follows some kind of pattern, not fully colocalizing with mTagBFP2. Why is mTagBFP2 so different in Fig 4A and Fig 4B? Is ER morphology affected by expression of HobΔN117-GFP? (KDEL-RFP puncta look smaller in Fig 4B than in Fig 4A). "Importantly, ER localization is critical for hobbit function, as ubiquitous overexpression of hobΔN117-GFP did not rescue the small body size or lethality of hobbit mutant animals": how do know this truncation does not have other effects beyond (presumably) affecting protein localization?

Our logic behind choosing the truncation site for Hobbit was the same as that described above for Fmp27. We have edited the text to make this clear. HobΔN117-GFP is a large protein that is unable to localize to the appropriate subcellular location; thus, it wouldn’t be surprising if this mutant protein aggregates, perhaps due to improper folding. Although the reviewer is correct in stating that HobΔN117-GFP does not fully co-localize with mTagBFP2, HobΔN117-GFP still diffuses out of the cell upon permeabilization with digitonin, as shown below. This confirms that N-terminally truncated Hobbit does indeed localize to the cytosol.

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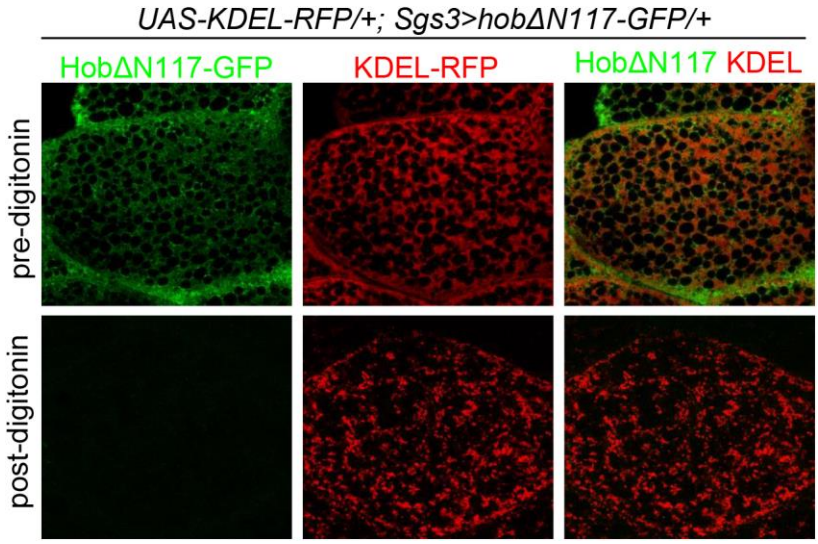

- The same applies to Fig S3B: how can we conclude from these images that HobbitΔC-GFP localizes to the ER? Also, it is unclear how the truncation site for Fmp27 was chosen (Fig S4A).

Because full-length Hobbit co-localizes with KDEL when imaged at lower magnification/resolution, as shown above, and HobΔC82 exhibits a similar pattern to full-length Hobbit when imaged at high resolution, this data does support the ER membrane localization of HobΔC82.

Additionally, as described above, we chose the C-terminal Fmp27 truncation site based on a gap in conservation of primary sequence. Since the C-terminus is quite conserved, the first significant gap was 595 amino acids from the C-terminus, leading us to generate Fmp27ΔC595.

- Fig 4C suggests that the protein is inserted in some intracellular membrane, not necessarily ER.

The reviewer is correct; however, the data we have described above in our previously published paper and in Fig. 4A shows that Hobbit localizes to the ER. Therefore, the protease protection assay serves as an additional layer of confirmation that Hobbit is anchored in the ER membrane.

- Fig 5: again, I am not familiar with this system, but how do we know those puncta are at the PM? "Additionally, StimDDAA-GFP appeared to localize normally in hobbit mutant cells (Fig. 5A)": those mutants have not yet explained in the text nor in the figure legend. Altogether, the ER/ER-PM localization of hobbit needs to be demonstrated more clearly.

As mentioned earlier, the salivary glands are composed of very large, 100 µm diameter cells. This allows us to easily image proteins and subcellular structures with a clear idea of spatial distribution. The images shown in Fig. 5 were acquired as z-stacks beginning outside of the cells of the gland and extending inward, through the plasma membrane and about 5 µm into the cell. Thus, we can easily see that the Stim^{DDAA}/Hob puncta are at the plasma membrane. We have also relabeled the images as “top view” and “side view” and edited the figure legend to add additional clarity. Finally, we have added text in the Results section to introduce the *hobbit* mutant alleles/animals.

- Please show the Pfam predictions, as well as the sequence comparisons with Atg2 and Vps13.

We have added a new supplemental figure (S5) that shows an alignment between the annotated Apt1 domain of Hobbit, Atg2, and Vps13. As you can see, the annotated Apt1 domain of Atg2 and Vps13 is much shorter than the Hobbit Apt1 domain, but there are several areas of highly conserved sequence.

- It would be important to test binding of Apt1 to other PIs: is there selectivity for any specific PI? The text says "These lipid moieties are known to be enriched at the plasma membrane", but PIs are present at many other membranes. Without understanding if there is any PI specificity, we

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cannot conclude that "ER-PM localization of Hobbit may be mediated by binding to plasma membrane phosphatidylinositols". "Fly Hobbit Apt1 bound to PI, PI(4)P, PI(4,5)P2, and PI(3,4,5)P3, while yeast Vps13 bound to PI(3)P and human VPS13A bound to PI(3)P and PI(5)P (Kolakowski et al., 2020; Rzepnikowska et al., 2017). Thus, Apt1 domains may represent a novel family of phosphatidylinositol binding domains with varying specificity": how can this comparison be established if binding to PI(3)P and PI(5)P was not tested for hobbit?

We have done this experiment and found that the Hobbit Apt1 domain binds to all PIs, but not to any other membrane lipids (see revised Fig. 7A). We have revised the Results and Discussion text to better fit these new results.

- "However, our data presented here suggests that the molecular function of hobbit lies in the regulation of phosphatidylinositol homeostasis": I do not think the data shown in Fig 7B suffice to establish the main function of hobbit.

We agree that there is still much to learn about the molecular function of *hobbit*. Hobbit is a large protein that is structurally uncharacterized. Our studies in this manuscript highlight the lipid binding properties of the Apt1 domain as critical for *hobbit* function, and we have revised the manuscript text to make this point clear.

Minor comments

Summary
- "Higher animals" should be substituted by "Drosophila".

We have edited the Abstract to make this change.

Introduction
- "ER-PM contact sites are particularly ubiquitous and prevalent in yeast": define "yeast" as "S. cerevisiae". There are of course other yeast species, but the following text refers only to S. cerevisiae.

We have edited the text to make the requested change.

"However, surprisingly, ER-PM contact sites are not required for yeast viability under normal laboratory growth conditions": it's worth mentioning that ER-PM contact sites are important under stress conditions such as heat shock (e.g. PMID 26864629, 31743662).

We have added this statement and references to the Introduction.

- Conservation: to what extent are Hobbit proteins conserved in vertebrates?

The Hob proteins are conserved throughout eukaryotes, from yeast to humans. We have also previously shown that the human ortholog of *hobbit* can rescue *Drosophila* mutants, suggesting that the protein is functionally conserved. We have added this information to the Introduction.

Results
- Perhaps it would make sense to rename Fmp27, as "Found in Mitochondrial Proteome" doesn't seem appropriate in the light of this manuscript.

While we agree with this statement, changing the name of yeast genes unfortunately goes against normal procedures for yeast genetics.

- Fig 6 is mentioned in the text before Fig 5.

We realize that this may be an annoyance for some readers, but we have chosen to leave the organization of the figures as is so that all of the rescue data remains grouped together to avoid generating partial figures and unnecessarily showing repetitive controls.

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Reviewer #2 (Significance (Required)):

In principle the findings reported here will be of interest to a broad cell biology audience. However, these findings need to be substantiated better before final publication, as described above. It would also be interesting to describe in more detail the conservation of Hobbit proteins in vertebrates, and the possible roles they may play there.

My expertise: structure of membrane contact sites.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

The authors present a straightforward series of experiments (done in yeast and flies) that characterizes Hobbit proteins as occupants of ER-PM contact sites. The data are of good quality and the conclusions are clear: yeast and fly hobbit are ER-anchored proteins with a lumenal N-terminus and extensive C-terminus, the last portion of which comprises a phosphoinositide-binding Apt1 domain that associates the protein with the PM and is needed for function. Of interest, without the Apt1 domain, the localization of phosphoinositides at the PM is dysregulated. I have some minor points for the authors to attend to, but also a significant comment about their unsupported claims that Hobbit could play a role in intermembrane lipid transfer.

Page 3, three lines from the end of the second paragraph, & page 4, 5 lines from the end of the top paragraph: there is no evidence that ER-PM contact sites play a role in sterol transfer/trafficking in yeast. In fact, the opinion article by Dittman/Menon (TIBS 2017) and the experimental test of this claim by Quon et al. (2018) indicate the opposite.

We appreciate the reviewer bringing this to our attention. We have revised the text accordingly.

Page 5, last sentence of Introduction: there is no evidence to support the claim that Hobbit may be a lipid transfer protein.

We have edited the text throughout the manuscript to make it clear that a lipid transfer function for *hobbit* is speculative at this point, and that future experiments will be required to directly test this possible function.

Page 8, Fig 3B: representative images should be shown, at least in the supplement.

We have added these images as requested in Fig. S1D.

Page 10, Fig 4A and 4B: enlarged views (such as shown in Figure 7) are needed as it is impossible to clearly see the described morphology. This could be done by providing insets.

We are admittedly a little confused by this comment. The images shown in Fig. 4A-B are already shown at very high magnification and were acquired at high resolution. The salivary glands are composed of large (~100 µm diameter) cells, which allows us to differentiate between the ER lumen and the ER membrane, at least at this developmental stage. Our response to Reviewer 2, above, provides further details and clarification about the ER membrane localization of Hobbit.

Fig 4C: it is surprising that the digitonin permeabilization method works well in drosophila cells given that they may not be as sterol-rich as mammalian cells. The authors may want to comment on this.

Digitonin permeabilization has previously been used in a variety of *Drosophila* cell lines and tissues (e.g. Ostroy et al 1974; von Stockum et al 2011), including the larval salivary glands (Tilly et al 2021). Additionally, our unpublished data indicates that the plasma membrane of the glands contains a significant amount of cholesterol. Thus, we were not surprised that digitonin permeabilization was effective for our protease protection assay experiments.

Fig S4: the expression level of the deletion mutant is much less than that of the full-length protein. A comment would be appropriate.

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We have added a statement about this to the appropriate figure legend.

Page 14: While reading I was at a loss to know what the Apt1 domain was. This becomes clear in the discussion section. A short explanation would be useful in the results, with a parenthetical statement such as 'see discussion for more detail'.

We thank the reviewer for bringing this up. We have added more detail to the Results section as well as a statement directing the readers to the Discussion section for additional information.

Discussion, last sentence first para: there is no evidence that Hobbit is a lipid trafficking regulator. The authors have shown that it affects the distribution of steady state pools of phosphoinositide. This could be the result of many factors.
Discussion, page 16, para 3: This sentence is also not justified - there are no data to support it (see comment above). "Given that Hobbit binds to phosphatidylinositols and that PI(4,5)P2 distribution appears to be altered in hobbit mutant cells, our data suggests that hobbit may be a novel lipid transfer protein at ER-PM contact sites"
Discussion, page 17, top para, last sentence: again not justified - see above.

As mentioned above, we have edited the text to make it clear that we are speculating on a possible lipid transfer function for the Hobbit protein.

Reviewer #3 (Significance (Required)):

This paper describes interesting new results on a new protein at ER-PM contact sites. The protein appears to localize to these sites without contributing a tethering function, and plays a role in regulating the distribution of phosphoinositides between the PM and an intercellular compartment. The authors over- reach by deducing that Hobbit is a lipid transfer protein - they have no data to support this claim. While undoubtedly interesting, the data represent just the first steps in looking into these proteins and therefore seem a little preliminary. Consequently, the paper could just as easily be published in a number of other journals, for example Mol Biol of the Cell or J Biol Chem.

Original submission

First decision letter

MS ID#: JOCES/2021/259086

MS TITLE: The Hob proteins are novel and conserved lipid binding proteins at ER-PM contact site

AUTHORS: Sarah D Neuman, Jeff R Jorgensen, Amy T Cavanagh, Jeremy T Smyth, Jane E Selegue, Scott David Emr, and Arash Bashirullah

ARTICLE TYPE: Research Article

Thank you for sending your manuscript to Journal of Cell Science through Review Commons.

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks. Thank you for submitting this interesting paper to JCS! Very interesting to learn about this new ER-PM contact protein and its unique lipid binding functions. Congratulations on these important discoveries and an excellent manuscript.

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