XK is a partner for VPS13A: A molecular link between Chorea-Acanthocytosis and McLeod syndrome

Jae-Sook Park and Aaron Neiman

Corresponding author(s): Aaron Neiman, State University of New York

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)
RE: E19-08-0439-T
TITLE: XK is an adaptor for VPS13A: A molecular link between Chorea-Acanthocytosis and McLeod syndrome

Dear Aaron,

Your paper entitled “XK is an adaptor for VPS13A: A molecular link between Chorea-Acanthocytosis and McLeod syndrome" has now been examined by two expert reviewers. As you will see both reviewers thought the work was interesting and the topic appropriate for MBoC, however they each had significant concerns about the interpretation of the data and specific suggestions for additional work to improve the strength of your conclusions and account for differences between your observations and previous work. While I'm not sure I agree with reviewer #2 (point 1) that you necessarily need to demonstrate direct binding in order to suggest XK is an adaptor for VPS13A, but the reviewer does offer an alternative explanation that I think should be considered and perhaps tested. Both reviewers expressed concerns with interpretation of Figure 6 and both have significant concerns in the interpretation of how mutations in VPS13A relate to localization changes and to the proposed model for the etiology of disease.

If you endeavor to address the major concerns of the reviewers then I would look forward to receiving a revised version with a cover letter that addresses each of the reviewers' comments point by point.

Sincerely,

Patrick Brennwald
Associate Editor

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Dear Dr. Neiman:

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made. Any specific areas to be addressed are outlined in the reviewer comments included below.

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Figure Size. Prepare figures at the size they are to be published.

1 column wide: Figure width should be 4.23-8.47 cm
1 to 1.5 columns wide: Figure width should be 10.16-13.3 cm
Reviewer #1 (Remarks to the Author):

The manuscript by Park and Neiman addresses the role of XK as an adaptor for VPS13A and the potential role this interaction plays in Chorea Acanthocytosis and McLeod Syndrome. Previous work in yeast demonstrated the role of adaptor proteins in mediating Vps13 recruitment to organelle contact sites. Here the authors show that XK interacts with VPS13A and that overexpression of XK leads to relocalization of VPS13A from lipid droplets to the ER and ER-mitochondrial contact sites. The authors then demonstrate that introduction of a disease associated missense mutation, W2460R, into VPS13A leads to mislocalization and the inability of XK overexpression to relocalize VPS13A. Experiments for the most part are straight forward, well-controlled, and easy to interpret. However, there are significant differences between the results shown by the authors and previous studies which need to be addressed. For example the authors find VPS13A to be predominantly localized to the lipid droplet absent XK overexpression, while others have shown predominant localization to ER-mitochondrial structures without XK overexpression. Similarly the final conclusion that the VAB domain is essential for proper VPS13A localization is in direct contrast to the finding by others that C-terminal domains lacking this VAB domain is sufficient for localization to ER-mitochondrial contact sites. While not mutually exclusive the authors need to address a potential mechanism by which disruption of the VAB domain might alter the ability of the C-terminal domain to localize to the ER-Mitochondrial contact site.

Major:

1. Kumar et al 2018 and Yenshaw et al 2019 describe localization of VPS13A to elongated structures as well as cytoplasmic puncta in some cells "By far the majority of the VPS13A signal occurred in the form of small patches arranged to form elongated structures". Additionally these publications demonstrate localization of VPS13A to Mitochondrial-ER structures in addition to Lipid Droplets in contrast to the conclusion drawn by the author in figures 3 and 5 which indicate 99% of VPS13A associates with LD in absence of XK overexpression. This discrepancy needs to be addressed.
2. Figure 4C, D, and F indicate GFP-XK(371-444) does not colocalize with Vps13A structures, images should be included to demonstrate that expression of this construct in measured cells is consistent with GFP-XK especially as Fig 1C indicates expression may be severely decreased.

3. Figure 5 demonstrates expression of GFP-XK leads to a reduction in VPS13A colocalization with lipid droplets. The authors indicate that expression of GFP-XK therefore releases VPS13A from lipid droplets. Notably however their images in Figure 5 appear to show a marked decrease in lipid droplet number and size which may indicate loss of lipid droplets is forcing relocalization of Vps13A. The relative number and size of lipid droplets should be reported if there is a significant change or a more representative image chosen if there is not.

4. Control experiments are needed in Figure 6 to quantitate relative changes in localization of VPS13A with lipid droplets, ER, and mitochondria in the absence of GFP-XK.

5. Mitochondria in Figure 6 appear to have an abnormal phenotype suggesting GFP-XK overexpression may be affected mitochondrial dynamics. The authors need to indicate whether this is a phenotype seen consistently and if so account for the possibility that effects on mitochondria may contribute to localization of VPS13A.

6. In Figure 7 the authors demonstrate that expression of VPS13A containing a mutation in the VAB domain at residue 2460 leads to mislocalization and localization is no longer affected by expression of GFP-XK. The authors indicate that therefore the VAB domain is essential for proper VPS13A localization.

This is in direct contrast to the findings by Kumar et al 2018 and Yeshaw et al 2019 where the C-terminal domain of VPS13A (2751-3174) and (2615-3174) respectively were shown to be sufficient for localization to both mitochondria and lipid droplets. Importantly these regions do not include the mutation generated by the authors. One possibility is that the C-terminal domain is regulated by the VAB domain however it's also possible that the mutation utilized may lead to protein misfolding of the C-terminal region thereby casting doubt on the author's interpretation.

Additionally in figure 7A the entire blot should be shown to indicate whether the mutation is leading to degradation of the protein which would indicate instability.

The author's argument would be strengthened if expression of the VPS13A C-terminal fragments utilized in Kumar et al 2018 and/or Yeshaw et al 2019 are unable to bind XK, supporting the notion that the VAB region is needed for this interaction and the mediated localization.

7. While Figure 7 shows a lack of punctate staining with XK-GFP there does appear to be some colocalization, this should be quantitated. Additionally IP experiments should be performed as in Figure 1 to determine whether the mutation has led to alterations in VPS13A-XK interactions or whether interactions are maintained but no longer lead to localization changes.

Minor:

1. The authors indicate that residues 425-435 of VPS13A contain the best match of the p-x-p motif and show that deletion does not abolish VPS13A-XK interactions. It should be stated how p-x-p consensus sequences were determined, whether this is the only significant match for a p-x-p motif, and if there are additional potential p-x-p motifs will need to adjust their claim that a p-x-p interaction is not required for Vps13a-XK interactions.

2. Figure 2 does not depict VPS13A, therefore VPS13A should be removed from the figure legend title and mentions of VPS13A replaced with GFP-XK.

3. While the author clearly demonstrates interactions between KX and Vps13A can occur in the absence of the C-terminal region (371-444) there is not enough evidence to conclude this region is not involved. In Figure 1 experiments are done with an N-terminal GFP tag which could alter activity of this domain. Ideally this experiment would be performed comparing C-terminally GFP tagged XK constructs. Additionally it is difficult to draw conclusions from this experiment as the c-terminal region is localized to a membrane (and anchored through a transmembrane domain) rather than cytosolic, suggesting a construct testing function of this domain should be at the minimum anchored to the membrane.

4. Any significance between constructs in 4C and 4F should be stated and potential significance of these differences should be indicated.

Reviewer #2 (Remarks to the Author):

The manuscript submitted by Park & Neiman sets out to provide a cell biological mechanism for the similar symptoms exhibited
in two diseases: Chorea-Acanthocytosis and McLeod syndrome. The causative genes for Chorea-Acanthocytosis and McLeod syndrome are known to be VPS13A and XK, respectively, with mutations in either gene leading to neurodegeneration. The study comes in the wake of Urata et al. (2019), who show that endogenous XK and VPS13A interact, and that VPS13A levels are reduced in patients with McLeod syndrome (lacking any detectable XK protein). Park and Neiman provide evidence for two major ideas: 1) that XK is an adaptor for VPS13A; 2) that the recruitment of VPS13A to membranes by XK is a causative link in the two diseases. If correct, their model would be an important mechanistic link between Chorea-Acanthocytosis and McLeod syndrome; however, currently their data do not fully support their claims. The nature of the interaction, its consequence on VPS13 localization, its relationship to disease-causing alleles of VPS13A, its relationship to known adaptors in yeast should be documented further.

**Major points**

1. If XK was a bona fide adaptor for VPS13A, the two proteins would be expected to directly interact with one another. The authors should therefore show direct interaction between the two proteins: for example, by yeast-two hybrid or pulldown studies using purified XK and VPS13A (e.g. GST-fusion or in vitro translated proteins). As XK has many transmembrane domains, the authors might want to consider using individual fragments of the cytoplasmic-facing sequences of XK. This would also serve to map the binding site for VPS13A, supporting the claim that a P-X-P motif is not required.

2. There are a few issues with the current data showing that XK can redistribute VPS13A within the cell. Firstly, in Figure 6, the authors propose that XK overexpression directly recruits VPS13A to puncta, to which both proteins localize. While this seems interesting, they also show that an unrelated ER protein is also recruited at the same place (and even better than XK, it seems), indicating that XK localization to these puncta is not specific, but is rather the result of a change in ER morphology. Therefore, an alternative interpretation is that XK overexpression causes accumulations of ER membranes to which XK and all other ER protein accumulate, and VPS13A associates with this abnormal ER. Secondly, the data in Figure 6 is used to suggest that VPS13A localises to ER-mitochondria contact sites upon overexpression of XK. The data here is far from convincing. Only 42% of the puncta were close to mitochondria. As the mitochondrial network is distributed extensively throughout the cell, it is possible that the VPS13A puncta observed would overlap with the mitochondria simply by chance. What would be the percentage of randomly-localized puncta that would overlap with mitochondria? Furthermore, how do the authors explain VPS13A only localising to one (or very few) ER-mitochondria contact sites?

3. More data is required to support the model that the XK-VPS13A interaction forms the aetiology of the two diseases. For example, the authors generate a VPS13A construct harbouring a disease-associated mutation, and while they have established pull-down assays, they do not test whether this mutant is impaired in its ability to interact with XK. They only rely on the fact that this mutant is not recruited to ER accumulations anymore. Yet, if our alternative interpretation is correct (see above), VPS13A might localize to these accumulation without necessarily interacting directly with XK. Additionally, the authors have previously published that disease-causing alleles of VPS13A engineered in yeast VPS13 caused mitochondrial-specific defects. In yeast, the mitochondrial specificity of Vps13 is bestowed by its interaction with the PxP domain of Mcp1. It is thus expected that disease-causing mutations somehow affect Vps13-PxP domain interaction. Yet this contradicts the present model that disease-causing mutations affect the non-PxP-dependent VPS13A-XK interaction. Finally, as McLeod syndrome can be caused by loss-of-function alleles, knockout or knockdown studies may also prove useful in supporting that the diseases are linked by their proposed model.

**Minor points**

1. The authors should consider shortening their introduction, as the current length dilutes some of their key concepts.

2. In a few places, the authors assert that the similar symptoms exhibited by the two diseases suggests that XK is an adaptor for VPS13A. The language here is too strong, as there are other plausible explanations for the similarities between the diseases.

3. “The α-VPS13A antibody was validated by its failure to detect VPS13A in HAP1 human cells containing a small internal deletion in the VPS13A gene (Figure 1A).”. This sentence is confusing. Rephrase?

4. The conclusion that VPS13A does not involve the PxP motif is overinterpreted. At best, the data suggest that the interaction is independent of the PxP motif that they have identified. However, the authors should consider that the human motif may have deviated from the PxP motif in yeast, and that a cognate motif exists in humans, but has escaped the bioinformatics search.

5. The image settings (or image processing) used in Figure 5 does not appear consistent between the two experiments. It could be that the localisation of VPS13A to lipid droplets in the GFP-XK overexpression condition is missed because the signal is dim in comparison to the bright VPS13A focus.
Dear Pat

Please find uploaded along with this letter a revised version of our manuscript "XK is an adaptor for VPS13A: A molecular link between Chorea-Acanthocytosis and McLeod syndrome". We thank the reviewers for their thoughtful comments and we have tried to respond to them as fully as possible. Several of the criticisms, for instance the apparent disparity in the localization of VPS13A^mCherry between our results and earlier reports, seemed to result from the low quality of several of the fluorescence micrographs in our original submission. This was due to our having collected data on the wide field microscope in my lab. As it turns out, that microscope is excellent for yeast work, but not so good for larger cells. We were fortunate to get access to a confocal microscope in a colleague's lab and have now repeated all of the microscopy experiments and replaced the micrographs in all of the figures with images obtained on the confocal microscope. The higher resolution and lower background with this instrument results in significantly clearer images. Importantly, the central conclusion of the manuscript is not changed by these new data.

One improvement with the confocal data is that the mutant VPS13A^W2460R protein can clearly be seen in the ER both in the presence and absence of XK overexpression (Figure 7). This not only strengthens the original conclusion that this mutant protein does not respond to XK, but addresses reviewer 2's concerns that all ER proteins were being non-specifically trapped in XK-generated structures.

In addition to the new confocal images, in response to reviewers, we have more carefully quantified the association of XK/VPS13A foci with mitochondria. Though there is a correlation, we do not find a statistically significant increase in the coincidence of VPS13A foci with mitochondria in the presence of XK. Accordingly, we have removed the conclusion that these foci represent mitochondrial-ER contacts from the paper.

One experiment that both reviewers suggested was a direct test of VPS13A^W2460R binding to XK via co-immunoprecipitation. As described below, we have tried multiple approaches and reagents to perform this experiment, but the experiment is not technically feasible with the antibodies and cell lines that we have available. Importantly, we do not claim that the W2460R mutation disrupts interaction with XK, only that the mutation influences the protein's localization pattern in vivo. While whether the mutation disrupts the co-IP would be a useful result to know, it is not essential for the conclusions in the paper.

We feel that the demonstration of an in vivo association of these two proteins implicated in related disease states is an important advance. I hope it will now be deemed acceptable for publication in MBoC.
Specific responses to reviewer comments (in blue text).

Reviewer #1 (Remarks to the Author):

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Major:

1. Kumar et al 2018 and Yenshaw et al 2019 describe localization of VPS13A to elongated structures as well as cytoplasmic puncta in some cells “By far the majority of the VPS13A signal occurred in the form of small patches arranged to form elongated structures”. Additionally these publications demonstrate localization of VPS13A to Mitochondrial-ER structures in addition to Lipid Droplets in contrast to the conclusion drawn by the author in figures 3 and 5 which indicate 99% of VPS13A associates with LD in absence of XK overexpression. This discrepancy needs to be addressed.

As explained above, some of the discrepancy between our images and the previously published results were caused by the difference in microscopes. In our images collected on the confocal the patchy distribution of Vps13A
throughout the ER that was previously reported is clearly visible. In addition, there is a misunderstanding here, as our data do not indicate that 99% of Vps13A associates with lipid droplets in the absence of XK overexpression but that 99% of the large, bright puncta of Vps13A (as opposed to the smaller dimmer patches distributed along the ER) co-localize with lipid droplet markers. We have tried to clarify this point in the text (p.9). The sentence the reviewer quotes from the earlier study notwithstanding, when we compare our images to those presented in the earlier papers, the abundance of Vps13A on lipid droplets does not seem to be very different in our hands.

2. Figure 4C,D, and F indicate GFP-XK(371-444) does not colocalize with Vps13A structures, images should be included to demonstrate that expression of this construct in measured cells is consistent with GFP-XK especially as Fig 1C indicates expression may be severely decreased.

We have now included a representative image of GFP-XK^{371-444} (new Figure 2A,iv), so that the distribution of the construct and level of fluorescence can be seen. The level of fluorescence is not as severely reduced as the protein appears to be on the Western blot.

3. Figure 5 demonstrates expression of GFP-XK leads to a reduction in VPS13A colocalization with lipid droplets. The authors indicate that expression of GFP-XK therefore releases VPS13A from lipid droplets. Notably however their images in Figure 5 appear to show a marked decrease in lipid droplet number and size which may indicate loss of lipid droplets is forcing relocalization of Vps13A. The relative number and size of lipid droplets should be reported if there is a significant change or a more representative image chosen if there is not.

We thank the reviewer for this thoughtful suggestion. We have quantified the abundance of lipid droplets in cells with and without XK transfection and found no significant difference. Moreover, lipid droplets are easily found in the GFP-XK VPS13A-mCherry co-transfected cells as can be seen in the new Figures 5 and 6.

4. Control experiments are needed in Figure 6 to quantitate relative changes in localization of VPS13A with lipid droplets, ER, and mitochondria in the absence of GFP-XK.

I’m not entirely sure I understand what the reviewer is requesting. The quantitation and control for the localization of Vps13A with lipid droplets is given in Figure 5. Vps13A is found in the ER with or without GFP-XK expression, but these larger foci, rings and lines are only seen when GFP-XK is overexpressed (quantified in Figure 4). For the mitochondrial co-localization the numbers have been added (p. 12, also see Reviewer 2 comment 2).
5. Mitochondria in Figure 6 appear to have an abnormal phenotype suggesting GFP-XK overexpression may be affected mitochondrial dynamics. The authors need to indicate whether this is a phenotype seen consistently and if so account for the possibility that effects on mitochondria may contribute to localization of VPS13A.

Again, the unusual appearance of the mitochondria had a lot to do with the microscope. In the confocal images, the distribution of mitochondria in XK-overexpressing cells appears unchanged from non-expressing cells. This can be seen in the representative image in the new Figure 6.

6. In Figure 7 the authors demonstrate that expression of VPS13A containing a mutation in the VAB domain at residue 2460 leads to mislocalization and localization is no longer affected by expression of GFP-XK. The authors indicate that therefore the VAB domain is essential for proper VPS13A localization.

This is in direct contrast to the findings by Kumar et al 2018 and Yeshaw et al 2019 where the C-terminal domain of VPS13A (2751-3174) and (2615-3174) respectively were shown to be sufficient for localization to both mitochondria and lipid droplets. Importantly these regions do not include the mutation generated by the authors. One possibility is that the C-terminal domain is regulated by the VAB domain however it’s also possible that the mutation utilized may lead to protein misfolding of the c-terminal region thereby casting doubt on the author’s interpretation.

We agree with the reviewer’s point that our results with the W2460R mutant are not fully consonant with the previously described localization of Vps13A protein fragments. The studies of protein fragments also need to be interpreted with care, as it is possible that protein fragments might bind to a specific organelle but are not sufficient to do so in the context of the full-length protein. We have expanded the discussion of these results to address these different possibilities (p. 15).

Additionally in figure 7A the entire blot should be shown to indicate whether the mutation is leading to degradation of the protein which would indicate instability.

We have repeated this experiment and include below a figure of the extended blot. As can be seen, there are a number of background cross-reacting bands at lower molecular weights, but there is no evidence of significant degradation of the mutant protein. Because all of the background bands detract from the figure when the fuller blot is shown, we have chosen to leave the reduced panel in Figure 7.
7. While Figure 7 shows a lack of punctate staining with XK-GFP there does appear to be some colocalization, this should be quantitated. Additionally IP experiments should be performed as in Figure 1 to determine whether the mutation has led to alterations in VPS13A-XK interactions or whether interactions are maintained but no longer lead to localization changes.

The confocal microscopy of the mutant Vps13A has revealed more clearly that the mutant protein is absent from the lipid droplets but localized throughout the ER and that this pattern is not altered by the expression of GFP-XK. A quantitative comparison of the overlap of the wild-type and mutant VPS13A with GFP-XK, along with an assessment of the statistical significance, has been added (p. 13).

We agree with the reviewer that testing the co-IP of XK with the mutant Vps13A would be valuable and we have tried multiple approaches to do this experiment. Unfortunately, we can’t use our ant-VPS13A antibodies for immunoprecipitation of the mutant protein because of the presence of endogenous Vps13A in the HEK293 cells. Both our GFP “specific” and mCherry “specific” reagents cross-reacted with the other fluorescent protein and no commercial XK antibodies we have tested proved specific for that protein. We also tried to do the experiment using the VPS13A knockout HAP1 cell line; however the transfection efficiency in those cells was so poor that we could not reliably precipitate the transfected protein (let alone test co-precipitation). Thus, for technical reasons we cannot address this issue at this time.

Minor:

1. The authors indicate that residues 425-435 of VPS13A contain the best match of the p-x-p motif and show that deletion does not abolish VPS13A-XK interactions. It should be stated how p-x-p consensus sequences were determined, whether this is the only significant match for a p-x-p motif, and if there are additional potential p-x-p motifs will need to adjust their claim that a p-x-p interaction is not required for Vps13a-XK interactions.

The logic for selecting 425-435 as the best candidate site is more fully explained on p. 7. Also, we have added discussion of the possibility that the mammalian interaction motif may be divergent from the PXP defined in yeast (p.15)

2. Figure 2 does not depict VPS13A, therefore VPS13A should be removed from the figure legend title and mentions of VPS13A replaced with GFP-XK.

Corrected

3. While the author clearly demonstrates interactions between KX and Vps13A can occur in the absence of the C-terminal region (371-444) there is not enough evidence to conclude this region is not involved. In Figure 1 experiments are
done with an N-terminal GFP tag which could alter activity of this domain. Ideally this experiment would be performed comparing C-terminally GFP tagged XK constructs. Additionally it is difficult to draw conclusions from this experiment as the c-terminal region is localized to a membrane (and anchored through a transmembrane domain) rather than cytosolic, suggesting a construct testing function of this domain should be at the minimum anchored to the membrane.

We agree with the reviewer that our data show only that the C-terminal region is not required, not that it plays no role. The text now states this clearly (p. 8). I am unclear on why a C-terminal GFP tag would be preferable for these experiments, as it seems to me better to have the free C-terminus. In any event, the only strong conclusion drawn in the paper is that the C-terminus is not essential for interaction.

4. Any significance between constructs in 4C and 4F should be stated and potential significance of these differences should be indicated.

Significant differences between the constructs and the control are noted on the figure, so I assume the reviewer is referring to potential significance of differences between the different XK fusions. Indeed, the difference between XK-GFP and the GFP-XK constructs in 4F is statistically significant (though the importance of this fact is not clear) and this is now noted in the text (p. 10).

Reviewer #2 (Remarks to the Author):

The manuscript submitted by Park & Neiman sets out to provide a cell biological mechanism for the similar symptoms exhibited in two diseases: Chorea-Acanthocytosis and McLeod syndrome. The causative genes for Chorea-Acanthocytosis and McLeod syndrome are known to be VPS13A and XK, respectively, with mutations in either gene leading to neurodegeneration. The study comes in the wake of Urata et al. (2019), who show that endogenous XK and VPS13A interact, and that VPS13A levels are reduced in patients with McLeod syndrome (lacking any detectable XK protein). Park and Neiman provide evidence for two major ideas: 1) that XK is an adaptor for VPS13A; 2) that the recruitment of VPS13A to membranes by XK is a causative link in the two diseases. If correct, their model would be an important mechanistic link between Chorea-Acanthocytosis and McLeod syndrome; however, currently their data do not fully support their claims. The nature of the interaction, its consequence on VPS13A localization, its relationship to disease-causing alleles of VPS13A, its relationship to known adaptors in yeast should be documented further.

Major points
1. If XK was a bona fide adaptor for VPS13A, the two proteins would be expected to directly interact with one another. The authors should therefore show direct
interaction between the two proteins: for example, by yeast-two hybrid or pulldown studies using purified XK and VPS13A (e.g. GST-fusion or in vitro translated proteins). As XK has many transmembrane domains, the authors might want to consider using individual fragments of the cytoplasmic-facing sequences of XK. This would also serve to map the binding site for VPS13A, supporting the claim that a P-X-P motif is not required.

We agree with the reviewer that mapping the interaction sites with direct assays is an important next step, but feel that this beyond the scope of this study.

2. There are a few issues with the current data showing that XK can redistribute VPS13A within the cell: Firstly, in Figure 6, the authors propose that XK overexpression directly recruits VPS13A to puncta, to which both proteins localize. While this seems interesting, they also show that an unrelated ER protein is also recruited at the same place (and even better than XK, it seems), indicating that XK localization to these puncta is not specific, but is rather the result of a change in ER morphology. Therefore, an alternative interpretation is that XK overexpression causes accumulations of ER membranes to which XK and all other ER protein accumulate, and VPS13A associates with this abnormal ER. Secondly, the data in Figure 6 is used to suggest that VPS13A localises to ER-mitochondria contact sites upon overexpression of XK. The data here is far from convincing. Only 42% of the puncta were close to mitochondria. As the mitochondrial network is distributed extensively throughout the cell, it is possible that the VPS13A puncta observed would overlap with the mitochondria simply by chance. What would be the percentage of randomly-localized puncta that would overlap with mitochondria? Furthermore, how do the authors explain VPS13A only localising to one (or very few) ER-mitochondria contact sites?

The reviewer raises two issues:

1) Is the colocalization of Vps13A and XK caused by changes of ER morphology rather than direct interaction? While this is possible, our improved images of the Vps13A mutant allele now show that, although the Vps13A mutant is present in the ER it is not concentrated in these sites. Therefore, not all ER proteins end up in these XK-containing structures.

2) Are these Vps13A/XK accumulations ER-mitochondria contact sites? The reviewer is correct to point out that controls for the quantitation of this colocalization were lacking in the original submission. We have now quantified the frequency of Vps13A colocalization with mitochondria in XK overexpressing and non-overxpressing cells (where the Vps13A foci are lipid droplets – a control of sorts for random association) both in our microscope and in the confocal. In both cases, there is an increase in colocalization of Vps13A with the mitochondrial marker in the presence of XK. However, the statistical significance is marginal (p=0.06). These data are now described in the Results (p.12). These results, therefore, do not
provide strong evidence that these VPS13A/XK foci are at contacts and, accordingly, we have removed the conclusion that VPS13A and XK interact at mitochondrial ER contacts. The issue of where these two proteins are co-localized is now considered in the Discussion (p.16).

3. More data is required to support the model that the XK-VPS13A interaction forms the aetiology of the two diseases. For example, the authors generate a VPS13A construct harbouring a disease-associated mutation, and while they have established pull-down assays, they do not test whether this mutant is impaired in its ability to interact with XK. They only rely on the fact that this mutant is not recruited to ER accumulations anymore. Yet, if our alternative interpretation is correct (see above), VPS13A might localize to these accumulation without necessarily interacting directly with XK. Additionally, the authors have previously published that disease-causing alleles of VPS13A engineered in yeast VPS13 caused mitochondrial-specific defects. In yeast, the mitochondrial specificity of Vps13 is bestowed by its interaction with the PxP domain of Mcp1. It is thus expected that disease-causing mutations somehow affect Vps13-PxP domain interaction. Yet this contradicts the present model that disease-causing mutations affect the non-PxP-dependent VPS13A-XK interaction. Finally, as McLeod syndrome can be caused by loss-of-function alleles, knockout or knockdown studies may also prove useful in supporting that the diseases are linked by their proposed model.

As noted in our response to reviewer #1, despite significant effort, for technical reasons we have been unable to examine the co-IP of the mutant protein with XK. However, as also noted above, the new micrographs of the mutant Vps13A protein demonstrate that the concentration of the wild-type Vps13A with XK in certain regions of the ER cannot simply be accounted for as an artifact of XK overexpression affecting all ER proteins.

While mutations that disrupt interaction with a mitochondrial-specific adaptor would be expected to cause mitochondrial defects that does not imply that those are the only type of mutations that could cause such defects. For example, the disease alleles of VPS13A engineered in yeast cause mitochondrial defects, but none of those alleles are in the VAB domain and would not be expected to alter binding to Mcp1. Thus, there is no contradiction in mutations that do not affect PxP binding displaying disease phenotypes.

Examining VPS13A localization in a XK knockout line is a useful suggestion but beyond the scope of the current study.

Minor points
1. The authors should consider shortening their introduction, as the current length dilutes some of their key concepts.
We have attempted to tighten up the text.

2. In a few places, the authors assert that the similar symptoms exhibited by the two diseases suggest that XK is an adaptor for VPS13A. The language here is too strong, as there are other plausible explanations for the similarities between the diseases.

We did not mean to imply that there are not other possible explanations. The last sentence of the Introduction (p. 6) has been rewritten to indicate that the similar symptoms suggest only that the two gene products may function in a similar pathway. The results presented indicate that XK may be an adaptor protein for VPS13A.

3. "The α-VPS13A antibody was validated by its failure to detect VPS13A in HAP1 human cells containing a small internal deletion in the VPS13A gene (Figure 1A)". This sentence is confusing. Rephrase?

Fixed.

4. The conclusion that VPS13A does not involve the PxP motif is overinterpreted. At best, the data suggest that the interaction is independent of the PxP motif that they have identified. However, the authors should consider that the human motif may have deviated from the PxP motif in yeast, and that a cognate motif exists in humans, but has escaped the bioinformatics search.

This possibility is now explicitly noted (p.15)

5. The image settings (or image processing) used in Figure 5 does not appear consistent between the two experiments. It could be that the localisation of VPS13A to lipid droplets in the GFP-XK overexpression condition is missed because the signal is dim in comparison to the bright VPS13A focus.

As noted, the manuscript now contains all new images obtained by confocal microscopy. We hope these are clearer.
Extended western blot corresponding to Figure 7 panel A:

Arrow indicates full length VPS13A
RE: E19-08-0439-TR
TITLE: XK is an adaptor for VPS13A: A molecular link between Chorea-Acanthocytosis and McLeod syndrome

Dear Aaron,

I sent your revised manuscript back to the same reviewers and as you will see below, the reviewers had quite different responses to your revisions. While Reviewer #1 was generally satisfied with your revisions, Reviewer #2 was not convinced that your revisions were sufficient to merit publication. This is tricky as I do think this reviewer's concerns are not unreasonable but I also think your results connecting these disease genes is unlikely to be only the result of an artifact due to overexpression/aggregation. Therefore, rather than rejecting the manuscript I am returning the manuscript to you with the suggestion that we will consider it further only with some additional experimental data to address the reviewers and my concern.

I consulted with reviewer #1 on possible remedies that would strengthen these analyses. This could include 1) additional work on the co-IPs comparing wt and mutant Vps13A. Perhaps using untagged Vps13A or untagged XK in the co-IP (as in Urata et al). 2) Additional analysis of the subcellular ER localization of Vps13A in the absence of XK. 3) Perhaps using lower amounts of DNA for transfection or use of a weaker promoter would allow you to characterize the ER localization (and effects of XK) at more physiological levels of Vps13A expression? 4) Additional co-localization data with mitochondria might determine if XK significantly enhances (or doesn't) the association of Vps13A with ER-mito contact sites.

I am sorry I can not bring you better news, but I hope you find the reviews helpful and that you use them to bring this study to a strong conclusion at MBoC or elsewhere.

Sincerely,

Patrick Brennwald
Associate Editor

Dear Dr. Neiman:

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made. Any specific areas to be addressed are outlined in the reviewer comments included below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision online please use the link below, and include a cover letter that details, point-by-point, how the Monitoring Editor's and reviewers comments have been addressed. When entering the author names online, enter them exactly as they appear on the manuscript title page. Please send only the latest revised manuscript. DO NOT resend any previous versions. Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

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Figure File Types. For revised manuscripts, figure files should be in .tif, .eps, or .pdf format. Files in .eps or .pdf formats must have their fonts embedded, and the images in them must meet the resolution requirements below.
Reviewer #1 (Remarks to the Author):

The revisions to the manuscript by Park and Neiman successfully addressed the majority of concerns identified in the original draft. Additionally, the large amount of effort involved to reproduce the figures with confocal microscopy is appreciated and greatly improves the manuscript. This manuscript is acceptable for publication pending changes to the text indicated below.

1. In the abstract and results (p. 11) the authors indicate that expression of XK leads to relocalization of VPS13A from LDs to ER subdomains, implying that VPS13A can move between the surface of LDs and the ER membrane. An alternative interpretation of the data is that VPS13A is always localized to an ER subdomain, which wraps around LDs in the absence of XK. In fact, this interpretation is consistent with correlative light EM data from Kumar et al. (2018), showing sec61B- and VPS13A- positive ER wrapped around LDs. The text should be adjusted to reflect this possibility.

Reviewer #2 (Remarks to the Author):

In their revised manuscript, Park and Neiman have used confocal microscopy to better support the claim that XK and VPS13A interact and that "XK is an adaptor for VPS13A". Unfortunately, other experiments that this and other reviewers felt necessary to make a solid point for this model, were either omitted or technically too challenging. In the end it is still not clear that XK is
indeed an adaptor for VPS13A. The evidence for XK-VPS13A interaction boils down to a coIP signal when both proteins are overexpressed. But no proper control show that this interaction is specific and not an artifact of overexpression. The other bit of evidence is that overexpression of VPS13A affects the localization pattern of XK and overexpression of XK, that of VPS13A. However, it I unclear whether these changes in localization pattern are specific. In the case of XK, it seems clear that the overexpression messes up ER structure, probably by causing aggregation on its surface.

The author claim that the XK-driven relocalization of VPS13A is specific because a mutant of VPS13 (W2460R) appears to be impervious to XK overexpression. However, what the mutation does to the protein is unclear, and while it is pretty clear that it destabilizes it, it is still far fetch to conclude that it prevents XK binding. It is just as likely to assume that overexpressed VPS13A and XK co-aggregate somewhere, affecting ER structure, and that the VPS13A(W2460R), which is much less expressed, is also much less prone to aggregation.

So in conclusions, the data are not solid enough to support the main claims of the paper.

Other points

Major:
- The ER localization of most proteins is not clear. E.g. Figure 3B is supposed to show ER accumulation of VPS13A, yet the image clearly show that VPS13A is present not on, but in between ER stacks and tubules. There is basically no overlap whatsoever. Also the authors claim that "In contrast, GFP-XK foci did not obviously correspond with the ER marker (Figure 2B vii - xii), indicating that these foci represent localization to some other compartment". However their ER marker is directed at the lumen while XK is at the membrane, thus these foci might represent membrane accumulations with little luminal content. Similarly, it is written that "VPS13AW2460R^mCherry was found distributed in small patches tubules that partially overlapped with an ER marker (Figures 7B and 3B)." Any ER staining is far from evident from these pictures. Moreover, the idea that VPS13AW2460R is found at the ER is contradictory with the "model that recruitment of VPS13A to lipid droplets (through an unknown adaptor) or to the ER (through XK) is mediated through the VAB domain". If XK recruits VPS13A to the ER but fails to do so for the W2460R mutant, then the mutant should not be on the ER.

Minor:
- There is an argument about whether N- or C-terminal tagging of XK might explain the discrepancy with a previous study. The conclusion appears that it does not explain it, and the discrepancy is still unexplained. Rearranging the paragraph starting with the C-terminally tag could save space and jump directly to the important conclusion.
Dear Pat

Please find uploaded along with this letter a revised version of our manuscript “XK is a partner for VPS13A: A molecular link between Chorea-Acanthocytosis and McLeod syndrome”. We appreciate the opportunity to submit a second revision and have tried to address the remaining concerns. Specifically, we have added experiments demonstrating that VPS13A carrying a mutation in the adaptor binding domain (VPS13A-W2460R) protein co-precipitates with the XK protein (new Figure 7A). This result is somewhat surprising given that the mutant protein no longer co-localizes with XK in ER subdomains. However, it is consistent with our other results that the sequences in XK related to the adaptor region binding motif identified in yeast are also dispensable for the interaction. Because XK does not appear to bind in the same way as the yeast “adaptor” proteins, we have changed the terminology throughout the paper to refer to XK as a VPS13 “partner”, rather than an “adaptor”. We have also added data (Figure 7E) showing that a second disease-associated allele of VPS13A present in a different domain of the protein similarly disrupts the co-localization of VPS13A with XK. This strengthens the correlation between loss of VPS13A localization with XK and the disease state. In addition, we repeated some of the co-localization experiments using Sec61 as the ER marker. Unfortunately, the quality of the images obtained using that marker was quite poor and so we have kept the original images.

I hope these changes go some way towards addressing the reviewer’s concerns and that the work will be considered acceptable for publication in MBoC.

Regards,

Aaron

Specific responses to reviewer comments:

Reviewer #1 (Remarks to the Author):

The revisions to the manuscript by Park and Neiman successfully addressed the majority of concerns identified in the original draft. Additionally, the large amount of effort involved to reproduce the figures with confocal microscopy is appreciated and greatly improves the manuscript. This manuscript is acceptable for publication pending changes to the text indicated below.

1. In the abstract and results (p. 11) the authors indicate that expression of XK leads to relocalization of VPS13A from LDs to ER subdomains, implying that VPS13A can move between the surface of LDs and the ER membrane. An alternative interpretation of the data is that VPS13A is always localized to an ER subdomain, which wraps around LDs in the absence of XK. In fact, this interpretation is consistent with correlative light EM data from Kumar et al.
(2018), showing sec61B- and VPS13A- positive ER wrapped around LDs. The text should be adjusted to reflect this possibility.

We thank the reviewer for this suggestion. This possibility is now included (p 11).

Reviewer #2 (Remarks to the Author):

In their revised manuscript, Park and Neiman have used confocal microscopy to better support the claim that XK and VPS13A interact and that "XK is an adaptor for VPS13A". Unfortunately, other experiments that this and other reviewers felt necessary to make a solid point for this model, were either omitted or technically too challenging. In the end it is still not clear that XK is indeed an adaptor for VPS13A. The evidence for XK-VPS13A interaction boils down to a coIP signal when both proteins are overexpressed. But no proper control show that this interaction is specific and not an artifact of overexpression. The other bit of evidence is that overexpression of VPS13A affects the localization pattern of XK and overexpression of XK, that of VPS13A. However, it I unclear whether these changes in localization pattern are specific. In the case of XK, it seems clear that the overexpression messes up ER structure, probably by causing aggregation on its surface.

The author claim that the XK-driven relocalization of VPS13A is specific because a mutant of VPS13 (W2460R) appears to be impervious to XK overexpression. However, what the mutation does to the protein is unclear, and while it is pretty clear that it destabilizes it, it is still far fetch to conclude that it prevents XK binding. It is just as likely to assume that overexpressed VPS13A and XK co-aggregate somewhere, affecting ER structure, and that the VPS13A(W2460R), which is much less expressed, is also much less prone to aggregation.

So in conclusions, the data are not solid enough to support the main claims of the paper.

The reviewer's underlying concern is that the interaction of Vps13 and XK that we see, as well as the co-localization are an artifact of overexpression and aggregation. Respectfully, we disagree with the reviewer. We think that this is unlikely to be an artifact for multiple reasons: 1) The W2460R protein does not co-localize but does co-precipitate with XK (new Figure 7A) – thus the two properties are separable and cannot both be caused by simple aggregation; 2) we now show (new Figure 7E) that a second disease allele of VPS13A also disrupts co-localization with XK; 3) an independent study has reported the co-precipitation using endogenous levels of expression; 4) a high throughput proteomic study identified the closely related XK paralog XKR2 as a VPS13A binding protein (now cited in Discussion). This is in addition to the biological phenotypes of mutants (ChAc and McCleod’s syndrome) that clearly link VPS13A and XK function. While for technical reasons we have not been able to do all of the experiments suggested, we believe that weight of evidence merits publication of our study.
Other points
Major:
-The ER localization of most proteins is not clear. E.g. Figure 3B is supposed to show ER accumulation of VPS13A, yet the image clearly show that VPS13A is present not on, but in between ER stacks and tubules. There is basically no overlap whatsoever.

The reviewer is correct that there is not perfect overlap of the two signals, but respectfully, there is clearly some coincidence of the signals in our images and these are consistent with the level of co-localization of VPS13A and ER markers shown in earlier work.

-Also the authors claim that "In contrast, GFP-XK foci did not obviously correspond with the ER marker (Figure 2B vii - xii), indicating that these foci represent localization to some other compartment". However their ER marker is directed at the lumen while XK is at the membrane, thus these foci might represent membrane accumulations with little luminal content.

As the reviewer suggests, the use of the luminal ER marker might explain some of the 'adjacent but not fully overlapping' localization patterns that we see. We repeated many of the localization experiments using Sec61 as the ER marker, but found that the results were very similar but the quality of the image was much lower than with the ER lumen marker. We, therefore, have kept the original images and have rewritten the description to include the possibility that these localizations are elaborations of ER (p. 9).

-Similarly, it is written that "VPS13AW2460R^mCherry was found distributed in small patches tubules that partially overlapped with an ER marker (Figures 7B and 3B)." Any ER staining is far from evident from these pictures.

See comment above

-Moreover, the idea that VPS13AW2460R is found at the ER is contradictory with the "model that recruitment of VPS13A to lipid droplets (through an unknown adaptor) or to the ER (through XK) is mediated through the VAB domain". If XK recruits VPS13A to the ER but fails to do so for the W2460R mutant, then the mutant should not be on the ER.

The new results with the W2460R co-precipitation, strongly indicate that the VAB domain is not responsible for interaction with XK. The statement cited was in the context of a model that additional proteins might mediate VPS13A localization to the ER and that these proteins don't necessarily bind through the VAB domain. Hopefully, avoiding the yeast terminology "adaptor" will make this clearer.
Minor:
- There is an argument about whether N- or C-terminal tagging of XK might explain the discrepancy with a previous study. The conclusion appears that it does not explain it, and the discrepancy is still unexplained. Rearranging the paragraph starting with the C-terminally tag could save space and jump directly to the important conclusion.

The paragraph has been revised (p.9).
Dear Aaron,

I have looked carefully over your 2nd revisions for your manuscript entitled "XK is a partner for VPS13A: A molecular link between Chorea-Acanthocytosis and McLeod syndrome" and I am happy to say that I think you did an admirable job in addressing the critiques of the previous reviews. Therefore I am recommending your paper be accepted at MBoC without any further revisions.

Congratulations,

Patrick Brennwald
Associate Editor
Molecular Biology of the Cell

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Dear Dr. Neiman:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

Within approximately four weeks you will receive a PDF page proof of your article.

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