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Peer Review Information

Journal: Structural and Molecular Biology
Manuscript Title: NSMB-A45178A _Chapman_Article_Handover.docx
Corresponding author name(s): Dr Edwin Chapman

Reviewer Comments & Decisions:

Decision Letter, initial version:

18th Aug 2021

Dear Ed,

Thank you again for submitting your manuscript "The complexin C-terminal amphipathic helix stabilizes the fusion pore open state by sculpting membranes". I apologize for the delay in responding, which resulted from the difficulty in obtaining suitable referee reports. Nevertheless, we now have comments (below) from the 3 reviewers who evaluated your paper. In light of those reports, we remain interested in your study and would like to see your response to the comments of the referees, in the form of a revised manuscript.

You will see that the reviewers, all experts in molecular neuroscience, are positive about the interest of the findings and the quality of the work. However, some revisions were requested. Reviewer #1 suggests that it should be attempted to strengthen the connection to the fusogenic properties of Cpx at the synapse, and asks for mutagenesis to confirm the role of the amphipathic properties of the CTD for the observed effects. Reviewer #3 requests additional controls and clarifications for the experiments in GUVs. The reviewer also asks for clarification of some points and has suggestions for a few additional experiments. Finally, all referees have useful suggestions to improve the presentation and discussion of the findings.

Please be sure to address/respond to all concerns of the referees in full in a point-by-point response and highlight all changes in the revised manuscript text file. If you have comments that are intended for editors only, please include those in a separate cover letter.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

We expect to see your revised manuscript within 6 weeks. If you cannot send it within this time, please contact us to discuss an extension; we would still consider your revision, provided that no similar work has been accepted for publication at NSMB or published elsewhere.

As you already know, we put great emphasis on ensuring that the methods and statistics reported in our papers are correct and accurate. As such, if there are any changes that should be reported, please submit an updated version of the Reporting Summary along with your revision.

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When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-research/editorial-policies/image-integrity">Digital Image Integrity Guidelines.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

SOURCE DATA: we urge authors to provide, in tabular form, the data underlying the graphical representations used in figures. This is to further increase transparency in data reporting, as detailed in this editorial (http://www.nature.com/nsmb/journal/v22/n10/full/nsmb.3110.html). Spreadsheets can be submitted in excel format. Only one (1) file per figure is permitted; thus, for multi-paneled figures, the source data for each panel should be clearly labeled in the Excel file; alternately the data can be provided as multiple, clearly labeled sheets in an Excel file. When submitting files, the title field should indicate which figure the source data pertains to. We encourage our authors to provide source data at the revision stage, so that they are part of the peer-review process.

Data availability: this journal strongly supports public availability of data. All data used in accepted papers should be available via a public data repository, or alternatively, as Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. Please note that for some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories can be found below:
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While we encourage the use of color in preparing figures, please note that this will incur a charge to partially defray the cost of printing. Information about color charges can be found at http://www.nature.com/nsmb/authors/submit/index.html#costs

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[REDACTED]

Note: This URL links to your confidential home page and associated information about manuscripts you may have submitted, or that you are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Kind regards,
Florian

Florian Ullrich, Ph.D.
Associate Editor
Nature Structural & Molecular Biology
ORCID 0000-0002-1153-2040

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
Summary

This study by Courtney and colleagues uncovers a surprising and novel physiochemical feature of the critical SNARE regulatory protein Complexin (Cpx) and explores some implications of this property in the context of an in vitro model of a SNARE-mediated bilayer fusion pore. This is an excellent research group with a long history of contributions to our molecular understanding of numerous synaptic proteins, and the results are of general interest to researchers studying the mechanisms underlying synaptic transmission and its regulation.

The basic result is an observation that the C-terminal domain of mammalian Cpx2 disrupts bilayer integrity facilitating leakage of dyes and electrical current as well as generating tubulation and vesiculation of liposomes. While the Cpx CTD has long been known to interact with membranes in a curvature-sensitive fashion (see Malsam 2009, Wragg 2013, Snead 2014, Gong 2016 for examples of both mammalian and invertebrate Cpx), the idea that the CTD can strongly remodel and disrupt bilayer structure is novel and fascinating. The authors characterize this feature of CTD through an impressive variety of methods (electrophysiology, fluorescence imaging, cryoEM, molecular dynamics) and speculate that this feature could underlie some of the fusogenic properties of Cpx at the synapse.

Strengths

The quantitative measures of ‘dye leakage’ and nanodisc fusion are very nice and convincing. The cryoEM is striking and shows us something that’s never been observed before with Cpx to my knowledge. A careful assessment of the membrane-perturbing characteristics of a major synaptic regulatory protein is an important contribution to the field and certainly worthy of publication. Comparisons between Cpx variants of different species are great since the field has been stymied by differences in the literature between vertebrate and invertebrate Cpx with few studies offering direct comparisons.

Weaknesses

1. If a major take-home message is that the membrane-perturbing properties of mCpx2 contribute to its role in synaptic vesicle fusogenicity, there isn’t a rock-solid connection being made here. In the nanodisc fusion pore assay, the presence of membrane-anchored Cpx causes a modest decrease in the pore

closing rate (seems like roughly 2-fold) and doesn't budge the opening rate constant. Supplemental Figure 6 perhaps has the most direct data with a SUV lipid mixing assay showing a 30-40% increase in the initial rate of mixing when mCpx2 is included (and this boosting is entirely CTD dependent). Do the authors have any data with a smaller copy number of vSNAREs? I am wondering if the briefer open pore dwell times could be extended when TMD-Cpx is then included. Maybe with 5 vSNAREs on the ND, the pore open state is already fairly stable and its harder to observe a stabilizing effect of the Cpx CTD. I still find the study interesting and compelling without this connection, so I wouldn't consider it to be a deal breaker - just perhaps oversold give this data set. I have the same issue with the title of this manuscript.

2. Another aspect that could be strengthened is the connection between the amphipathic properties of the CTD and the observed effects on dye leakage, nanodisc fusion, and SUV lipid mixing. I like the CpxDelta21 controls, but it would have been more convincing to use point mutants that disrupt some amphipathic property or maybe some secondary structure that is key for its AMP-like character. The authors substitute a couple hydroxyamino acids for aspartate in Supp Fig 5 where the dye leak is abrogated by a ST/DD variant. Does this have a similar effect on the ND fusion assay, cryoEM, MD sims, etc…? And what is the AMP score when you feed in the sequence with the ST/DD substitution into the SVM model? Would this substitution be predicted to radically change those sorts of properties? If so, this insight should definitely be highlighted. Another example of testing Cpx specificity would be to use the CpxDelta21-melittin (from Fig S1) on a TMR anchor in the ND fusion to ask if this can also stabilize fusion pores. Not that all of these possibilities would need to be tested for this current study, but a little stronger connection between specific properties of the Cpx CTD and its activity would bolster the claims made here.

Minor Issues

3. The cartoon in Figure 5 appears to depict Cpx as sitting in a parallel orientation on the SNARE complex (N- and C- termini in the same orientation). But in reality, crystal structures indicate that Cpx sits in an anti-parallel orientation on the SNARE bundle (Chen 2002 for example). Am I thinking about this incorrectly, or is this just an artistic accident? – could be misleading for readers trying to think about the orientation of the C-terminal domain.

4. I'm curious as to why there were no controls with FL TMR-Cpx harboring the SBMs in the ND fusion assay. That way, one can study the impact of the CTD in the absence of SNARE binding. Maybe the SNARE binding was preventing some of the impact of the CTD on fusion pore initiation or stability? Or the CTD somehow antagonizes the central helix clamping effect on the SNAREs.

5. The authors observe that fly 7A failed to disrupt membrane integrity while fly 7B was similar to mCpx2. If this property is proposed to be related to the fusogenic aspects of Cpx function, the authors should note that 7B had a stronger fusogenic function than 7A with 200% EPSC amplitude in Buhl 2013,

similar to mCpx1. This is evidence supporting the authors' current hypothesis. On a related note, the authors claim that 7A has no amphipathic helix, but this claim may be false. There's a reasonable AH sequence just upstream from the CAAX box: FTTKLKKRLSDAFKNCPL. How does this score in the SVM model? Note that many CAAX box proteins possess a hydrophobic or amphipathic helix just upstream of the CAAX where the farnesyl transferase enzyme binds. And when using the fly Cpx 7A variant, I assume the authors left the CAAX residues in the purified protein, whereas in vivo, the last three residues would be gone, and the cysteine would be prenylated. This could have a large effect on its lipid-interacting properties but didn't seem to be considered.

6. Given the authors' previous fascinating discovery regarding the dynamic assembly/disassembly of the SNAREs in the ND fusion pore assay (Bao), were there any experiments done here using the cd-Syb2 competition in the presence of TMR-Cpx? It could be interesting to show potential mCpx impact on SNARE complex disassembly or if the CTD effects this.

Reviewer #2:
Remarks to the Author:
This expertly crafted manuscript reports a striking membrane-remodeling activity associated with a C-terminal amphipathic helix of complexin. Complexin, despite its small size, has been one of the most mechanistically mysterious of the proteins that play central roles in neurotransmitter release. The discovery and characterization of its membrane-remodeling activity is therefore likely, in my judgment, to be a game-changing advance in deciphering its mechanism of action.

Previous work had suggested that a complexin C-terminal amphipathic helix binds membranes and might play a role in vesicle tethering. The authors instead discover that the C-terminal helix is necessary and sufficient to form transmembrane pores. Since punching holes in membranes is clearly not complexin's physiological function, the authors go on to use an impressive array of methods (and controls) to investigate complexin's membrane remodeling activities. Molecular dynamics simulations suggest that, at lower 'copy numbers', complexin might have pore-stabilizing activity. To pursue this, they return to the system they have developed previously, in which v-SNARE-containing nanodiscs "fuse" with t-SNARE-containing black lipid membranes (BLMs) to form pores whose current can be measured. Complexin was initially deemed incompatible with this system because it singlehandedly forms pores in the BLM. As a clever alternative, the authors add an artificial anchor, along with a flexible linker, and thereby incorporate complexin at low copy number into the nanodiscs. This strategy allows them to directly measure the effect of complexin on the lifetime of the open and closed states of the SNARE-mediated fusion pore. They find a dramatic 10-fold stabilizing effect of complexin on the open state, leading to the conclusion that complexin's stimulatory function in neurotransmitter release derives from its ability to be recruited to trans-SNARE complexes where it stabilizes the fusion pore.

Briefly, this system also allows the authors to decouple complexin's two activities, SNARE "clamping" and fusion pore stabilization.

Another key player, synaptotagmin, is not studied here but also interacts with trans-SNARE complexes and inserts into the membranes. Elucidating the complex architecture of the fusion pore will doubtless be a worthy and challenging goal for future research. Another major goal of future research will be to figure out what protects cells against complexin's pore-forming activity. Nonetheless, this manuscript feels like a suitably complete story and is, in my view, publishable essentially as is.

I only have minor quibbles. The authors argue (lines 209-219) that "the closing rate of TMD-Cps pores were significantly shorter as compared to the syb2 alone control" but the data weren't very convincing. Also I think there is a mixup in the right versus left panels of Fig. 5g.

Reviewer #3:
Remarks to the Author:
Review on the manuscript entitled "The complexin C-terminal amphipathic helix stabilizes the fusion pore open state by sculpting membranes" by Courtney et al.

In this manuscript, Courtney et al, focus on the role of the C terminal region of Complexin (here, Complexin II, noted Cpx). Complexins I and II are short proteins that have been thoroughly studied in the past and identified as key players in the regulation of SNARE induced fusion during neurotransmission. They have somewhat contradictory simultaneous functions of facilitator and inhibitor of fusion. For instance, they can facilitate fusion by increasing the distance at which cognate SNAREs can find each other and assemble. They also hinder the fusion process by preventing the SNAREpins to fully assemble. In addition, they may modulate fusion regulation because they simultaneously interact with Synaptotagmin 1 and the SNAREpins. In view of the considerable amount of work that has been devoted to Cpx, it may seem that everything is known about this protein. This is incorrect because the currently identified functions are induced by two domains of the protein, the central helix and the accessory helix. The role, if any, of the C-terminal region has never been previously determined. This manuscript nicely shows how this C-terminal region interacts with lipids to promote and/or stabilize curved regions of membranes. This is an important result that definitely deserves to be published because it implies that, unexpectedly, Cpx also stabilizes the fusion pore itself and possibly can act on its SNARE-induced formation.

The membrane sculpting ability of Cpx is nicely shown in an experimental assay that the authors master in which pores open in a black lipid membrane. In a related assay (nanodisc-black lipid membrane fusion), they also show that SNARE induced fusion pores are stabilized by the C-terminal region of Cpx.

The simulations of the interactions between Cpx and lipid membranes support very well the experimental results. This forms a convincing set of results that can almost be published as is.

The vesiculation part is much less convincing, notably the GUVs experiments. They may even somewhat reduce the strength of the case the authors are making here. Perhaps it would be appropriate to remove them or only keep the extended data figure S1 (please see comments below).

Specific comments:

1.Vesiculation
Figure 3b (Cpx and SUMO-Ct21) and the supplementary movie look like artifacts. This type of behavior is typically observed on GUVs in presence of glycerol because of the transient negative osmotic difference between the lumen of the GUV and the outer medium containing glycerol. This osmotic difference disappears either when glycerol crosses the membrane, typically over a few seconds, or when the membrane breaks apart before resealing (as seen here). The fact that this is not observed below 1µM tends to support this interpretation. On the other hand, the DeltaCt21 variant is comforting but more statistics are needed and a single image is not sufficient to really make a good control because the local protein (and glycerol?) concentration may locally vary a lot after injection.

a. Before anything the authors need to confirm that there is no glycerol in these experiments.

b. If there is glycerol, they absolutely need to repeat the experiments without glycerol. Alternately they could just focus on the lower concentrations where glycerol is more diluted (currently presented in Fig S1). This is sufficient to prove the pore forming capability of CPX.

c. If there is no glycerol, these observations are still difficult to interpret because the resulting vesicles in Fig 3 and the movie are still giant (1-15 µm). This seems in contradiction with the TEM pictures. The authors need to explain why CPX does not continue reducing the GUV size down to the 30 nm particle size observed in TEM. What is stopping the reaction?

d. Minor: The kinetics of the of the fluorescence increase could be better analyzed in terms of diffusion. What is the gyration radius of JF635i? What is the concentration in bulk of JF635i? How can these gyration radius and concentration explain such a fast increase of fluorescence in 10 s? Is it quantitatively consistent with the diffusion of the dye?

2. Pore forming activity of Cpx
The experiments and the simulations form a very convincing set of data. There may be some ways to make the results a bit more quantitative.

a. Can the authors estimate an equivalent Cpx concentration in the simulations and compare it with the experiments?

b. Why does the Cpx pore reseal? Is it due to a low resealing energy barrier or to the removal of one or more peptides? This may be discussed in view of the lifetime distributions at various concentrations (below 500 nM).

c. SUMO-Ct21 displays transient pores of smaller size than Cpx at 500 nM. What could explain this behavior?

d. When zooming in around the transient pores, is a plateau observed? If so, fluctuations around the plateau value would provide direct information on the energetics of the pore.

e. The HEK patch-clamp experiments (pore formation in the plasma membrane) are not fully conclusive. How do the authors explain that in 14 out of 21 trials there was no event observed with WT Cpx?

f. Regulation of the pore forming activity by phosphorylation and Calmodulin. Because of the issues on the GUV experiments, experiments at 1µM Cpx or less would be more appropriate, or, even better, electric measurements just like Fig. 1. Also, it is not clear what the white pixels represent in the GUVs (Fig. S5b)

g. Minor. It would be helpful to show (draw) the connection between the pore itself (Fig. 2b, 12 peptides) and the various diameters presented in Fig 2.e. It is hard to see the 7 nm when 1nm away from the minimum pore diameter. For instance, a few arrows showing the various diameters on one of the pictures may be a good solution.

3. Stabilization of the SNARE-induced fusion pore. This part is nicely done; no comment.

4. Minor. Please, carefully proofread the manuscript. Here are a few typos/missing details but there are probably many more:
a. Line 120 Fig 3a not 1a
b. Line 121 and 124: Fig 3 not 1
c. Fig. S5e, no need for x 10^3 for an arbitrary unit.

Mat. and Met.
d. Lines 12, 17 and 24 ° has to be indicated instead of the current symbol
e. Line 35: What chains (PC and PS not sufficient)?
f. Line 48: Fig. 3

g. Line 59: For extrusion it is always an odd number to avoid recovering the initial aggregates. Is it really 50?
h. Line 220: is it really 5µm?
i. ND-Black Lipid membrane description is confusing. Why start with SNAREs when it is only used at the very end?

Frédéric Pincet

Author Rebuttal to Initial comments

Response to reviewer comments

Reviewer #1:

Remarks to the Author:

Summary

This study by Courtney and colleagues uncovers a surprising and novel physiochemical feature of the critical SNARE regulatory protein Complexin (Cpx) and explores some implications of this property in the context of an in vitro model of a SNARE-mediated bilayer fusion pore. This is an excellent research group with a long history of contributions to our molecular understanding of numerous synaptic proteins, and the results are of general interest to researchers studying the mechanisms underlying synaptic transmission and its regulation.

The basic result is an observation that the C-terminal domain of mammalian Cpx2 disrupts bilayer integrity facilitating leakage of dyes and electrical current as well as generating tubulation and vesiculation of liposomes. While the Cpx CTD has long been known to interact with membranes in a curvature-sensitive fashion (see Malsam 2009, Wragg 2013, Snead 2014, Gong 2016 for examples of both mammalian and invertebrate Cpx), the idea that the CTD can strongly remodel and disrupt bilayer structure is novel and fascinating. The authors characterize this feature of CTD through an impressive variety of methods (electrophysiology, fluorescence imaging, cryoEM, molecular dynamics) and speculate that this feature could underlie some of the fusogenic properties of Cpx at the synapse.

Strengths

The quantitative measures of 'dye leakage' and nanodisc fusion are very nice and convincing. The cryoEM is striking and shows us something that's never been observed before with Cpx to my knowledge. A careful assessment of the membrane-perturbing characteristics of a major synaptic regulatory protein is an important contribution to the field and certainly worthy of publication. Comparisons between Cpx variants of different species are great since the field has been stymied by differences in the literature between vertebrate and invertebrate Cpx with few studies offering direct comparisons.

Weaknesses

1. If a major take-home message is that the membrane-perturbing properties of mCpx2 contribute to its role in synaptic vesicle fusogenicity, there isn't a rock-solid connection being made here. In the nanodisc fusion pore assay, the presence of membrane-anchored Cpx causes a modest decrease in the pore closing rate (seems like roughly 2-fold) and doesn't budge the opening rate constant. Supplemental Figure 6 perhaps has the most direct data with a SUV lipid mixing assay showing a 30-40% increase in the initial rate of mixing when mCpx2 is included (and this boosting is entirely CTD dependent). Do the authors have any data with a smaller copy number of vSNAREs? I am wondering if the briefer open pore dwell times could be extended when TMD-Cpx is then included. Maybe with 5 vSNAREs on the ND, the pore open state is already fairly stable and its harder to observe a stabilizing effect of the Cpx CTD. I still find the study interesting and compelling without this connection,
so I wouldn't consider it to be a deal breaker - just perhaps oversold give this data set. I have the same issue with the title of this manuscript.

Author response: We thank the reviewer for encouraging us to strengthen the connection between the membrane remodeling activity and the fusion pore stabilization properties of Cpx. This is indeed our take-home message. We have addressed this issue in our revised

manuscript by correcting a graphical error, described below, and by generating a new complete ND3 dataset that culminated in a new main figure (Fig. 5).

In the original version of our manuscript, we demonstrated that TMD-Cpx increased the open dwell time of the fusion pores by 10-fold (Fig. 5f, now Fig. 6c). We showed that TMD-Cpx reduced the fusion pore closing rate, compared to syb2 alone, while TMD-Cpx ΔCt21 dramatically increased the closing rate (Fig. 5g, now Fig. 6d). We also found that incorporation of TMD-Cpx did not alter the current passing through the pore, indicating that the pores may have reach a maximal dilation state in the ND5 condition, as suggested by the reviewer.

Both reviewer 1 and 2 found the pore-stabilizing effect of Cpx in our ND-BLM dataset to be underwhelming, as it appeared that Cpx only reduced the closing rate by 2-3 fold. Upon closer inspection of our original Fig 5g (now Fig. 6d, *left panel*), we noticed an error in how these data were plotted that masked the true effect. As shown below, a zoomed-in pairwise comparison of syb2 alone and syb2 + TMD-Cpx reveals that Cpx caused an approximate 8-fold change in the closing rates, on average (the average closing rate decreased from 0.00303 to 0.0000371 (1/ms)). We now see that this 8-fold change was masked by the y-axis offset in the original version of the figure (Fig. R1, *left panel*), giving the false impression of a 2-3 fold change in the closing rate between syb2 alone and syb2 + TMD-Cpx. Although the data are unchanged, removing the misleading y-axis offset makes the Cpx-mediated 8-fold reduction in the average fusion pore closing rate far more apparent (Fig. R1, *right panel)*. We are grateful that the reviewer brought this to our attention and have modified the representation of this figure to avoid further confusion.

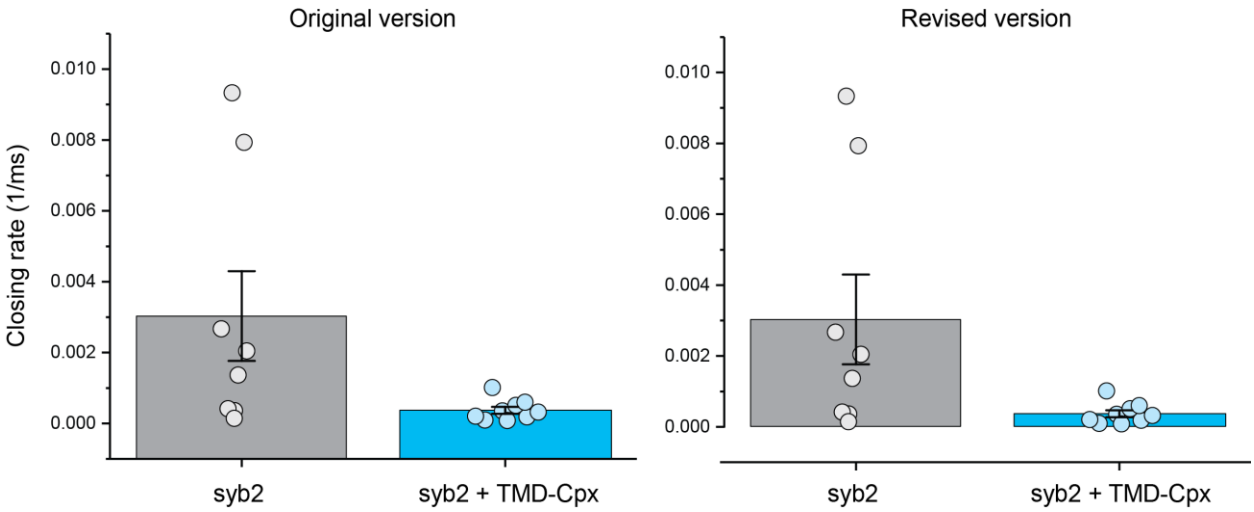

Fig. R1. Zoomed-in comparison of the original (left panel) and revised (right panel) versions of Fig. 5g in the original manuscript (now Fig. 6d). By starting the y-axis at -0.001, the representation of the data in the original bar graph was misleading. The revised figure now more accurately represents the closing rate data.

Although correcting the graphical misrepresentation in Fig. 5g (Now Fig. 6d) may have already resolved the issues raised in the above comment, we found the reviewer's lower copy number hypothesis intriguing and proceeded to test nanodiscs with three copies of syb2 (ND3). As previously observed[1], we found that ND3 created small, unstable fusion pores in the t-SNARE-containing BLM. Like the ND5 condition, we found that incorporation of three copies of TMD-Cpx into the nanodiscs resulted in a significant stabilization of the fusion pore open dwell time. The closing rate was again significantly reduced 13-fold by TMD-Cpx (as compared to the 8-fold change using ND5, detailed above). However, in striking contrast to the ND5 condition, we found the ND3 TMD-Cpx condition displayed a significant 3.5-fold increase in the current passing through the pore, indicative of a larger pore; the pore opening rate was also significantly increased 4-fold in this condition. Conversely, TMD-Cpx ΔCt21 failed to increase the current or stability of the pore. The results from this series of experiments perfectly matched the reviewer's hypothesis. Since these experiments provide strong support of our initial conclusion and demonstrate a new result (that Cpx can both stabilize the pore and increase the pore size) we have created a new main figure to summarize these results, reproduced below.

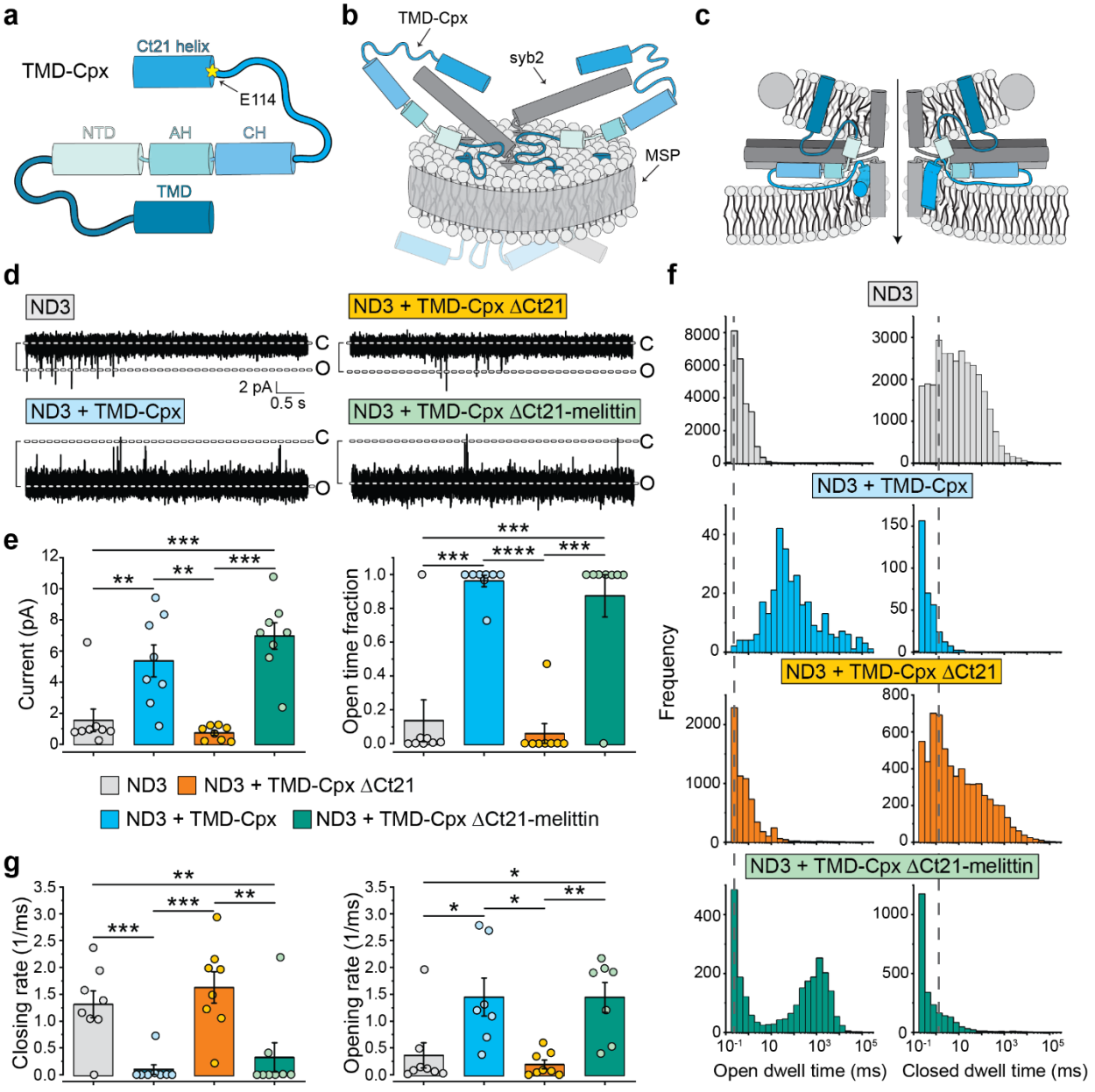

Fig. R2. Reproduction of the new complete ND3 dataset. TMD-Cpx experiments were repeated with ND3 and included in the revised manuscript as a new Fig. 5.

2. Another aspect that could be strengthened is the connection between the amphipathic properties of the CTD and the observed effects on dye leakage, nanodisc fusion, and SUV lipid mixing. I like the CpxDelta21 controls, but it would have been more convincing to use point mutants that disrupt some amphipathic property or maybe some secondary structure that is key for its AMP-like character. The authors substitute a couple hydroxyamino acids for aspartate in Supp Fig 5 where the dye leak is abrogated by a ST/DD variant. Does this have a similar effect on the ND fusion assay, cryoEM, MD sims, etc…? And what is the AMP score when you feed in the sequence with the ST/DD substitution into the SVM model? Would this substitution be predicted to radically change those sorts of properties? If so, this insight should definitely be highlighted. Another example of testing Cpx specificity would be to use the CpxDelta21-melittin (from Fig S1) on a TMR anchor in the ND fusion to ask if this can also stabilize fusion pores. Not that all of these possibilities would need to be tested for this current study, but a little stronger connection between specific properties of the Cpx CTD and its activity would bolster the claims made here.

Author response: Our manuscript demonstrates that Cpx can dramatically remodel phospholipid bilayers and stabilize the nascent fusion pore open state. We used planar lipid bilayer electrophysiology, coupled with a machine-learning algorithm, to identify Cpx residues E114-K134 (Ct21) as the crucial determinant for these effects. Moreover, both the remodeling activity and the fusion pore stabilization properties of Cpx were abrogated by truncation of Ct21 from the C-terminus. While a helical wheel projection suggests that Ct21 could form an amphipathic helix, we agree with the reviewer that a stronger connection could be made between the specific amphipathic properties of the Ct21 region and the observed effects of Cpx. The reviewer provided excellent suggestions for control experiments that would strengthen this aspect of our study.

Among these, the TMD-Cpx ΔCt21-melittin chimera is particularly interesting. We initially showed that Ct21 truncation blocked pore opening, but we did not perform any “truncation-rescue” experiments using the ND-BLM system in the original version of our manuscript.

Demonstrating that an alternative amphipathic helix (melittin) could rescue the Ct21 truncation defect would greatly strengthen our study and we thank the reviewer for this suggestion. Since melittin is, itself, a pore-forming molecule that likely requires four peptides or more for activity [2,3], we elected to generate ND3 nanodiscs with three copies of TMD-Cpx ΔCt21-melittin (1.5 copies per face of the nanodisc) for these experiments, rather than using an ND5 condition (thus avoiding melittin-mediated pores). To test if TMD-Cpx ΔCt21-melittin NDs could form melittin-mediated pores (ie. SNARE-independent pores), we incubated the ND3 TMD-Cpx ΔCt21-melittin sample with the BLM, in the absence of t-SNAREs. These experiments demonstrated that TMD-Cpx ΔCt21-melittin failed to generate pores, validating that the melittin copy number is below its intrinsic pore formation threshold. We have now included these results in a new Extended data figure (Extended data fig. 6) in the revised manuscript.

Upon incorporation of t-SNAREs into the BLM, we found that ND3 TMD-Cpx ΔCt21-melittin was able to generate SNARE-mediated pores, as expected. Strikingly, we found that TMD-Cpx ΔCt21-melittin increased the size and stability of the pores, compared to the syb2 alone condition, similar to the ND3 with TMD-Cpx. This result matches the reviewer's hypothesis that the amphipathic properties of the Ct21 helix can be generalized by substitution with alternative amphipathic residues. These results have now been incorporated into the ND3 dataset as a new Fig. 5.

In addition to the melittin chimera, the reviewer requested that we perform additional characterization of the Cpx C-terminal phosphomimetic mutant (S115, T119D). In our original manuscript, we demonstrated that the S115, T119D substitution reduced the pore forming activity of Cpx, as determined by experiments with our GUV leak assay. Comparable charge mutations S115D[4] or L117K[5] were also reported to block a Cpx1-mediated increase in SUV lipid-mixing assays. Much to our surprise, a posthoc AMP prediction query of the S115, T119D substitution had a negligible impact on the AMP prediction score (0.83 vs 0.82 for WT and S115, T119D, respectively); the algorithm failed to predict our experimental result. Curiously, when querying the L117K substitution, the algorithm predicted an increased pore-forming probability (0.91), yet this mutation was functionally impaired[5]. While the AMP prediction software enabled us to map the critical pore-forming residues of Cpx, validating the general utility of this software, the result of the S115, T119D and L117K substitution queries revealed a

limitation to the prediction algorithm. Clearly, the algorithm needs further refinement, so we are currently working with our collaborators to better understand and rectify this issue.

Despite this predictive limitation, we proceeded to further examine the functional consequences of the S115, T119D substitution by performing SUV-SUV lipid mixing assays. We performed lipid-mixing assays for this set of experiments, as it was impractical to address all the Cpx mutants using the ND-BLM system, since that assay is exceptionally time-consuming (note: we opted to use our revision time to include an ND3 dataset and to study the Cpx-melittin chimera in the BLM system). We generated a TMD anchored variant (TMD-Cpx (S115, T119)) and co-reconstituted the purified protein with syb2 into SUVs to perform lipid-mixing assays via fusion with t-SNARE SUVs. In our original manuscript, we demonstrated that TMD-Cpx caused a significant increase in the lipid-mixing rate between v- and t-SNARE SUVs. We repeated these experiments to compare the lipid-mixing between t-SNARE SUVs and SUVs containing syb2 alone, syb2 +TMD-Cpx or syb2 + TMD-Cpx (S115, T119D). As shown below, we found that the extent of lipid-mixing was significantly reduced by the S115, T119D substitution, compared to TMD-Cpx.

Please note that in this set of experiments, we found that the syb2 alone samples reached approximately 20% of the total lipid-mixing after 60 minutes, while our initial series of experiments found syb2 alone lipid-mixing reached close to 30%. This variability is attributed to the use of different materials between these experiments that were performed more than a year apart. Since these new syb2 alone data are not cohesive with our original dataset, and since S115D data have already be reported[4], we have elected to exclude the TMD-Cpx (S115, T119D) lipid mixing from our revised manuscript.

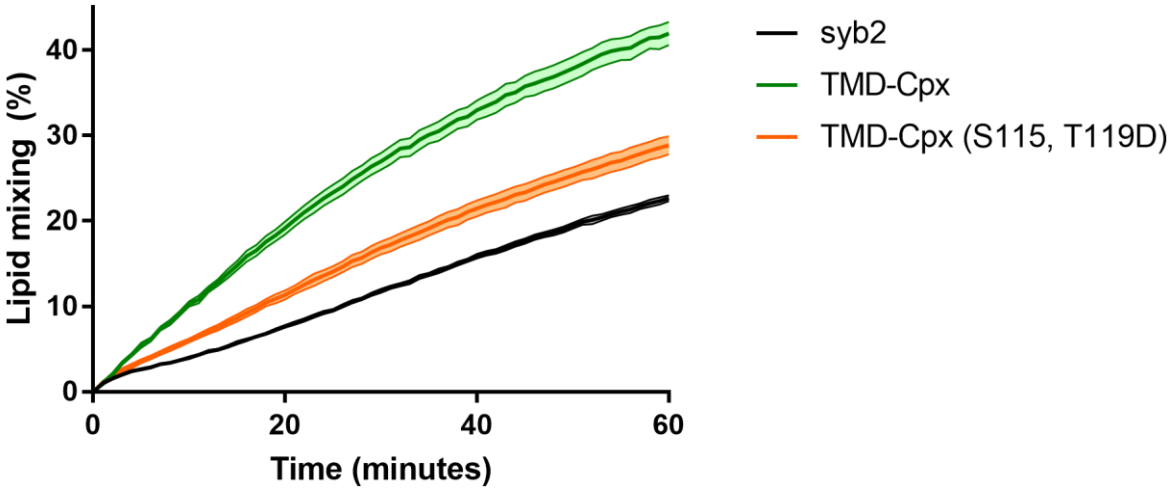

Fig. R3. Comparison of SUV-SUV lipid-mixing between syb2 alone, syb2 + TMD-Cpx and syb2 + TMD-Cpx (S115, T119D).

For completeness, we also sought to examine how the TMD-Cpx ΔCt21-melittin variant would perform in the SUV-SUV lipid-mixing assay. Unfortunately, after several attempts, we determined that proteoliposomes could not form in the presence of this chimera. As exemplified below, upon detergent removal, TMD-Cpx ΔCt21-melittin consistently produced large, inter-connected lipid aggregates that were incompatible with our assay. Interestingly, we did not experience any technical issues with TMD-Cpx ΔCt21-melittin reconstitution into nanodiscs, thus enabling the ND3 + TMD-Cpx ΔCt21-melittin dataset in the new Fig. 5.

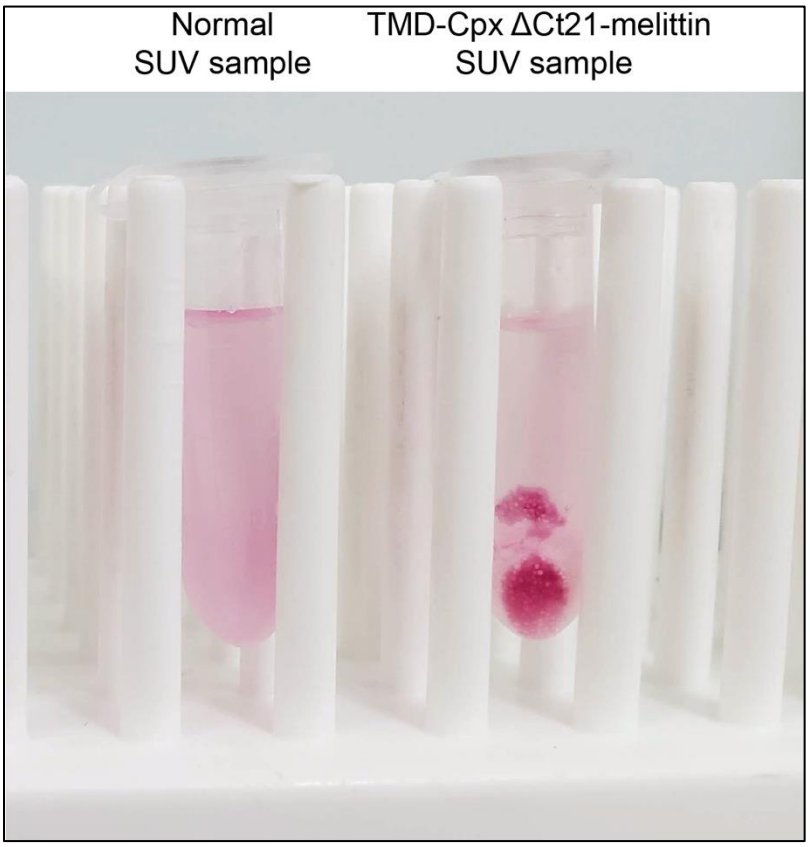

Fig. R4. TMD-Cpx ΔCt21-melittin cannot be reconstituted into small unilamellar vesicles.

Finally, we also produced a soluble variant of Cpx where the residues that comprise the Ct21 helix were randomly scrambled, rather than truncated. We converted the WT Ct21 sequence (ESILDTVLKYLPGPLQDMFKK) to YLQLKDLPKEVSGTPDLIKMF and performed GUV leakage assays using this variant. The scrambled sequence produced an AMP prediction score of 0.07, suggesting this construct would be unlikely to exhibit pore-forming ability. As predicted, our GUV leakage assay showed that TMD-Cpx Ct21 scrambled had no effect on the GUVs. These results are now included in the revised Extended data fig. S5, shown below.

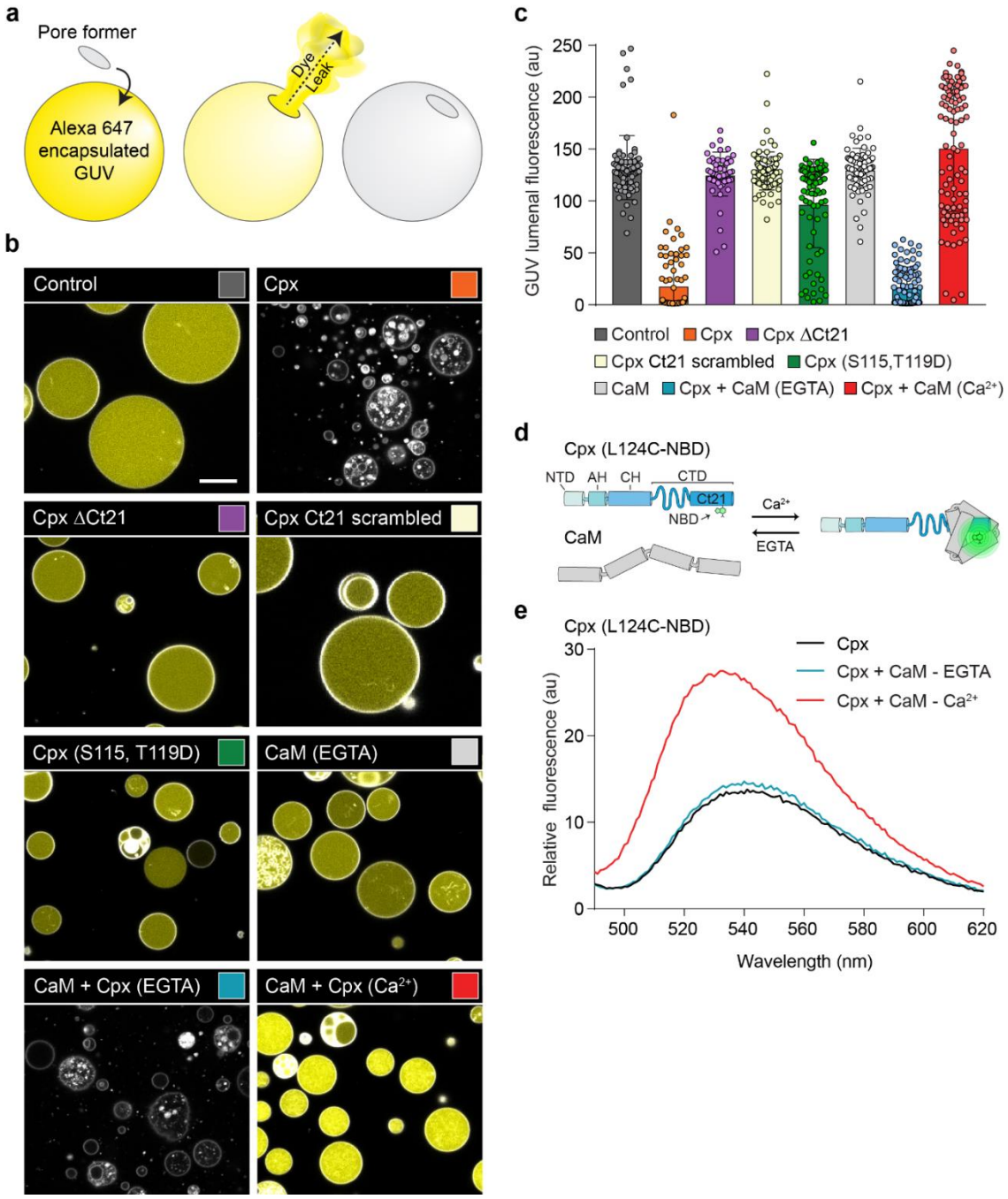

Fig. R5. Reproduction of the updated Extended data Fig. S5, including data using the Cpx Ct21 scramble construct.

In summary, the new data generated using the melittin chimera, the phosphomimetic and the Ct scrambled variant provide consistent evidence to further support our title claim that the Cpx C-terminal amphipathic helix stabilizes the nascent fusion pore open state by sculpting membranes. We are grateful for these constructive reviewer suggestions.

Minor Issues

3. The cartoon in Figure 5 appears to depict Cpx as sitting in a parallel orientation on the SNARE complex (N- and C- termini in the same orientation). But in reality, crystal structures indicate that Cpx sits in an anti-parallel orientation on the SNARE bundle (Chen 2002 for example). Am I thinking about this incorrectly, or is this just an artistic accident? – could be misleading for readers trying to think about the orientation of the C-terminal domain.

Author response: The cartoon depiction of Cpx bound to the SNARE complex in Fig. 5c was indeed an error; we thank the reviewer for catching this oversight. We have modified the cartoon (shown below) to represent Cpx bound to the SNARE complex in an anti-parallel fashion.

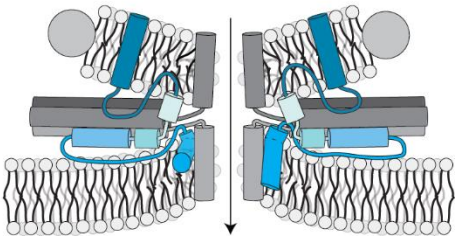

Fig. R6. A revised illustration from Fig. 5c showing the cross-section of a *trans*-SNARE complex formed between the TMD-Cpx ND and the t-SNARE BLM. The revised illustration depicts Cpx binding to the *trans*-SNARE complex in an anti-parallel orientation.

4. I'm curious as to why there were no controls with FL TMR-Cpx harboring the SBMs in the ND fusion assay. That way, one can study the impact of the CTD in the absence of SNARE binding. Maybe the SNARE binding was preventing some of the impact of the CTD on fusion pore

initiation or stability? Or the CTD somehow antagonizes the central helix clamping effect on the SNAREs.

Author response: The ND5 BLM experiments in our original manuscript showed that truncation of the Ct21 residues (Cpx ΔCt21) resulted in a dramatic reduction in fusion pore size and stability. These surprising results were indicative of this Cpx mutant acting as a robust fusion clamp. To validate that Cpx ΔCt21 may be acting directly upon the SNAREs to inhibit pore opening, we made additional mutations that are known to mediate SNARE binding (R48L,59H) and found that the fusion pores were again large and stable, similar to the syb2-alone condition. While the fusion pore clamping result is dramatic and interesting, the ultimate objective of our study was to examine the fusion promoting attributes of Cpx and how Cpx interacts with membranes. In our view, this dataset clearly demonstrates that Cpx can stabilize the fusion pore open state, mediated by the Ct21 helix. The new ND3 dataset further supports this conclusion. The FL-TMD-Cpx-SBM experiments, proposed by the reviewer, were omitted simply because this experiment is somewhat tangential to our initial objective. We believe that an examination of whether and how full-length Cpx can exhibit a clamping function in our ND-BLM system would be more appropriate for a planned follow-up study that incorporates both TMD-Cpx and synaptotagmin 1, as these two regulatory proteins appear to act in concert[6] to clamp or promote fusion. During the revision of our current manuscript, we did attempt to reconstitute the FL-Cpx (R48L,59H) into nanodiscs, however, we experienced technical challenges with reconstitution efficiency of this mutant. We will continue to work on this and are committed to addressing this complicated issue (of delving into the cryptic clamping activity of Cpx) in our next nanodisc-BLM study.

5. The authors observe that fly 7A failed to disrupt membrane integrity while fly 7B was similar to mCpx2. If this property is proposed to be related to the fusogenic aspects of Cpx function, the authors should note that 7B had a stronger fusogenic function than 7A with 200% EPSC amplitude in Buhl 2013, similar to mCpx1. This is evidence supporting the authors' current hypothesis. On a related note, the authors claim that 7A has no amphipathic helix, but this claim may be false. There's a reasonable AH sequence just upstream from the CAAX box:

FTTKLKKRLSDAFKNCPL. How does this score in the SVM model? Note that many CAAX box proteins possess a hydrophobic or amphipathic helix just upstream of the CAAX where the farnesyl transferase enzyme binds. And when using the fly Cpx 7A variant, I assume the authors left the CAAX residues in the purified protein, whereas in vivo, the last three residues would be gone, and the cysteine would be prenylated. This could have a large effect on its lipid-interacting properties but didn't seem to be considered.

Author response: We agree with the reviewer that results presented by Buhl et al (2013) are a nice complement to our experimental data. We appreciate this suggestion and have now referred to this study within our revised manuscript, as follows:

"The differential effects induced by *Dm*Cpx 7A and 7B in our GUV assay are also consistent with an *in vivo* report demonstrating that *Dm*Cpx 7B exhibited a two-fold increase in evoked neurotransmitter release, compared to *Dm*Cpx 7A."

Furthermore, we also appreciate the reviewer's suggestion to emphasize the functional difference of *Dm*Cpx 7A and 7B using the AMP prediction algorithm. Upon examination of the C-terminal sequences of *Dm*Cpx 7A and 7B, we note that the peptide sequence listed in the reviewer's comment, above, is derived from *Dm*Cpx 7B, not 7A. The putative C-terminal amphipathic helices of *Dm*Cpx 7A and 7B are TKLKNQIETQVNELKTQIEGK and FTTKLKKRLSDAFKNCPL, respectively. A direct comparison of these sequences can also be found in Fig. 3 of the Buhl et al. (2013) publication (doi:10.1016/j.mcn.2012.11.009). In line with our experimental data, the results of the AMP prediction queries report an antimicrobial peptide score of 0.43 and 0.99 for *Dm*Cpx 7A and *Dm*Cpx 7B, respectively. This further supports our experimental data, suggesting that the C-terminus of DmCpx 7A is unlikely to exhibit membrane remodeling activity. We now report the AMP prediction scores for *Dm*Cpx 7A and 7B in our revised manuscript.

Regarding the CAAX residues in recombinant *Dm*Cpx 7A, the reviewer is correct to assume that these residues remained on the C-terminus of our purified protein. Certainly, post-translational prenylation of *Dm*Cpx 7A will have a significant impact on the properties of the protein. A direct comparison of prenylated and non-prenylated *Dm*Cpx 7A in an *in vitro* system

could indeed be a fascinating follow-up study. In fact, we are currently developing a strategy to generate palmitoylated synaptotagmin isoforms for *in vitro* studies and this approach could be readily applied to Cpx prenylation. So, we will conduct these experiments in the future. We thank the reviewer for emphasizing this and agree that these details are relevant to our conclusions and discussion. We now include the following statement in the revised manuscript:

"It is also important to note that *Dm*Cpx 7A is prenylated *in vivo,* as this post-translational modification will greatly influence the membrane interaction properties of this isoform."

6. Given the authors' previous fascinating discovery regarding the dynamic assembly/disassembly of the SNAREs in the ND fusion pore assay (Bao), were there any experiments done here using the cd-Syb2 competition in the presence of TMR-Cpx? It could be interesting to show potential mCpx impact on SNARE complex disassembly or if the CTD effects this.

Author response: The proposed cd-syb2 experiments are an excellent suggestion. It would indeed be interesting to examine how Cpx affects SNARE complex disassembly and we fully intend to do this. We devoted the majority of our time during revision to generate the full ND3 dataset, including the TMD-Cpx ΔCt21-melittin experiments and were unfortunately unable to include cd-syb2 experiments in this study. We consider our current manuscript to be the first in a series of Cpx studies using the ND-BLM system. As described above, our follow-up study will increase in complexity by incorporating syb2, TMD-Cpx and synaptotagmin 1 into the nanodiscs. This follow-up study will delve further into SNARE complex disassembly by examining how Cpx influences the action of cd-syb2 and αSNAP/NSF, in comparison to synaptotagmin 1, as previously reported[7], and after incorporation of both Cpx and synaptotagmin 1.We expect this study to be quite interesting and we thank the reviewer for the suggestion.

Reviewer #2:

Remarks to the Author:

This expertly crafted manuscript reports a striking membrane-remodeling activity associated with a C-terminal amphipathic helix of complexin. Complexin, despite its small size, has been one of the most mechanistically mysterious of the proteins that play central roles in neurotransmitter release. The discovery and characterization of its membrane-remodeling activity is therefore likely, in my judgment, to be a game-changing advance in deciphering its mechanism of action.

Previous work had suggested that a complexin C-terminal amphipathic helix binds membranes and might play a role in vesicle tethering. The authors instead discover that the C-terminal helix is necessary and sufficient to form transmembrane pores. Since punching holes in membranes is clearly not complexin's physiological function, the authors go on to use an impressive array of methods (and controls) to investigate complexin's membrane remodeling activities. Molecular dynamics simulations suggest that, at lower 'copy numbers', complexin might have pore-stabilizing activity. To pursue this, they return to the system they have developed previously, in which v-SNARE-containing nanodiscs "fuse" with t-SNARE-containing black lipid membranes (BLMs) to form pores whose current can be measured. Complexin was initially deemed incompatible with this system because it singlehandedly forms pores in the BLM. As a clever alternative, the authors add an artificial anchor, along with a
flexible linker, and thereby incorporate complexin at low copy number into the nanodiscs. This strategy allows them to directly measure the effect of complexin on the lifetime of the open and closed states of the SNARE-mediated fusion pore. They find a dramatic 10-fold stabilizing effect of complexin on the open state, leading to the conclusion that complexin's stimulatory function in neurotransmitter release derives from its ability to be recruited to trans-SNARE complexes where it stabilizes the fusion pore. Briefly, this system also allows the authors to decouple complexin's two activities, SNARE "clamping" and fusion pore stabilization.

Another key player, synaptotagmin, is not studied here but also interacts with trans-SNARE complexes and inserts into the membranes. Elucidating the complex architecture of the fusion

pore will doubtless be a worthy and challenging goal for future research. Another major goal of future research will be to figure out what protects cells against complexin's pore-forming activity. Nonetheless, this manuscript feels like a suitably complete story and is, in my view, publishable essentially as is.

I only have minor quibbles. The authors argue (lines 209-219) that "the closing rate of TMD-Cps pores were significantly shorter as compared to the syb2 alone control" but the data weren't very convincing. Also I think there is a mixup in the right versus left panels of Fig. 5g.

Author response: As with the first question raised by reviewer 1 above, we greatly appreciate the encouragement to strengthen our title claim that the Cpx C-terminal amphipathic helix stabilizes the fusion pore open state. Please see our detailed response to reviewer #1 above, where we explained that the original version of Fig. 5g (now Fig. 6d) was misleading due to the y-axis offset. By extending the bar graph to meet the x-axis in the original version, the difference between syb2 alone and syb2 + TMD-Cpx was masked. When re-plotting the data in Fig. 5g (now Fig. 6d), the 8-fold change in the closing rates between these two conditions becomes more apparent.

We are grateful to both of the reviewers for bringing this matter to our attention and we have now updated the figure to correct this issue. Furthermore, as described in response to reviewer #1, our revised manuscript now includes a full dataset examining the effect of Cpx on fusion pore properties in an ND3 paradigm (three copies of syb2 or three copies of syb2 and TMD-Cpx). These ND3 experiments show that Cpx exerts an even greater effect on the fusion pore properties (average current increased 3.5-fold, average closing rate decreased 13-fold, average opening rate increased 4-fold and fraction of time open increased from almost 0 to almost 1), compared to our initial ND5 dataset. The ND3 results are summarized in a new main figure of the revised manuscript, and greatly strengthen our confidence in our initial conclusions.

Please also note that the panels in Fig. 5g in the original version of the manuscript were not inverted: While somewhat counterintuitive, the fusion pore closing rates are derived from the open dwell time CDF, and the opening rates are derived from the closed dwell time CDF. We fit the CDFs with an exponential function ($y = 1\text{-}e^{-Ax}$) to solve for these rates.

Initially these analyses were displayed in the figure by vertically aligning the rate quantification with the associated histograms, however, these data have now been re-organized in a revised Fig. 6, and are no longer vertically aligned. We expect this updated layout will be less ambiguous.

Additionally, the reviewer's comment also prompted us to perform some additional analyses of these data. Figures 5 and 6 now each include a new panel of data that reports the fraction of time open for the pores in each of the conditions used in this study. These data effectively demonstrate the effect of Cpx (and the subsequent mutations) on fusion pore properties and we thank the reviewer for sparking these additional analyses.

Reviewer #3:

Remarks to the Author:

Review on the manuscript entitled "The complexin C-terminal amphipathic helix stabilizes the fusion pore open state by sculpting membranes" by Courtney et al.

In this manuscript, Courtney et al, focus on the role of the C terminal region of Complexin (here, Complexin II, noted Cpx). Complexins I and II are short proteins that have been thoroughly studied in the past and identified as key players in the regulation of SNARE induced fusion during neurotransmission. They have somewhat contradictory simultaneous functions of facilitator and inhibitor of fusion. For instance, they can facilitate fusion by increasing the distance at which cognate SNAREs can find each other and assemble. They also hinder the fusion process by preventing the SNAREpins to fully assemble. In addition, they may modulate fusion regulation because they simultaneously interact with Synaptotagmin 1 and the SNAREpins. In view of the considerable amount of work that has been devoted to Cpx, it may seem that everything is known about this protein. This is incorrect because the currently identified functions are induced by two domains of the protein, the central helix and the accessory helix. The role, if any, of the C-terminal region has never been previously determined. This manuscript nicely shows how this C-terminal region interacts with lipids to promote and/or stabilize curved regions of membranes. This is an important result that definitely deserves to be published because it implies that, unexpectedly, Cpx also stabilizes the fusion

pore itself and possibly can act on its SNARE-induced formation.

The membrane sculpting ability of Cpx is nicely shown in an experimental assay that the authors master in which pores open in a black lipid membrane. In a related assay (nanodisc-black lipid membrane fusion), they also show that SNARE induced fusion pores are stabilized by the C-terminal region of Cpx. The simulations of the interactions between Cpx and lipid membranes support very well the experimental results. This forms a convincing set of results that can almost be published as is.

The vesiculation part is much less convincing, notably the GUVs experiments. They may even somewhat reduce the strength of the case the authors are making here. Perhaps it would be appropriate to remove them or only keep the extended data figure S1 (please see comments below).

Specific comments:

1.Vesiculation
Figure 3b (Cpx and SUMO-Ct21) and the supplementary movie look like artifacts. This type of behavior is typically observed on GUVs in presence of glycerol because of the transient negative osmotic difference between the lumen of the GUV and the outer medium containing glycerol. This osmotic difference disappears either when glycerol crosses the membrane, typically over a few seconds, or when the membrane breaks apart before resealing (as seen here). The fact that this is not observed below 1µM tends to support this interpretation. On the other hand, the DeltaCt21 variant is comforting but more statistics are needed and a single image is not sufficient to really make a good control because the local protein (and glycerol?) concentration may locally vary a lot after injection.

a. Before anything the authors need to confirm that there is no glycerol in these experiments.

b. If there is glycerol, they absolutely need to repeat the experiments without glycerol.

Alternately they could just focus on the lower concentrations where glycerol is more diluted (currently presented in Fig S1). This is sufficient to prove the pore forming capability of CPX.

Author response: We thank Dr. Pincet for the critical interpretation of our GUV results. Indeed, GUV experiments can be prone to artifacts in some contexts. For example, many GUV studies have reported experiments that were performed in the absence of buffered saline solutions (i.e. sucrose in water). In our case, the GUV experiments are performed in 20 mM HEPES, 100 mM KCl (pH 7.4), with the osmolarity of the buffers used for the internal and external solutions equalized and validated using an osmometer.

We agree that glycerol itself can affect GUV morphology in a concentration dependent manner. We have first-hand experience with this and have taken steps to avoid such artifacts. Our stock purified proteins contain 5% glycerol. However, prior to use in the GUV experiments, aliquots of the protein samples were passed through a PD-10 column that was equilibrated with glycerol-free GUV imaging buffer. This ensured that our observed effects were specific to the proteins in the sample, rather than an artifact of the buffer. We regret to have omitted these details in our initial submission. We have now included this information in the Methods section. On a related note, we emphasize that various control experiments were performed (shown in Extended data fig. S1 and S5), and these demonstrate that the observed membrane remodeling activity is specific to Cpx. We emphasize that all of these proteins were in the exact same buffer, but only Cpx constructs with an intact C-terminal amphipathic helix altered the structure of GUVs. Moreover, Extended data fig. S5 demonstrates that calmodulin (CaM) can bind Cpx in the presence of Ca^{2+} and prevent the membrane remodeling activity. These CaM competition experiments contain three times the amount of recombinant protein pipetted into the sample (10 µM CaM and 5 µM Cpx), yet GUVs remained unaffected. Only upon addition of EGTA, to chelate Ca^{2+}, does Cpx become free to act upon the GUVs. Together, these data strongly support the conclusion that Cpx can dramatically alter phospholipid bilayer morphology.

c. If there is no glycerol, these observations are still difficult to interpret because the resulting vesicles in Fig 3 and the movie are still giant (1-15 µm). This seems in contradiction with the

TEM pictures. The authors need to explain why CPX does not continue reducing the GUV size down to the 30 nm particle size observed in TEM. What is stopping the reaction?

Author response: The apparent contradiction between the GUV imaging, via fluorescence microscopy, and the 100 nm LUV imaging by TEM can be rectified by considering the relative size of the two vesicles, the imaging method and the concentrations used in the two experiments. From a technical perspective, the generation of 30 nm vesicles from the GUV surface is not resolvable by fluorescence microscopy; such tiny vesicles appear as diffraction limited puncta, even when using super-resolution Zeiss Airyscan imaging (120 nm spatial resolution). In fact, this is what initially led us to perform TEM imaging. As shown below, TEM imaging demonstrates that treating GUVs with 10 µM Cpx does indeed generate ~30 nm structures.

Additionally, the GUV fluorescence microscopy that we reported in our manuscript was performed using 2-5 µM Cpx, while the TEM and Cryo-EM experiments were performed using 10 µM Cpx. As demonstrated in Extended data fig. S1, the Cpx remodeling effect is concentration dependent. The Cpx concentrations vary between experiments in this study, depending on the phenomenon that we aim to demonstrate. Specifically, when applying less than 1 µM Cpx, only pores are observed (no vesiculation). When the concentration increases above 2 µM, moderate vesiculation occurs, but many of the GUVs remain within the resolution of our fluorescence microscope. As the Cpx concentration reaches 10 µM, the effect on liposomes is quite dramatic and completely reduces the vesicles to ~30 nm structures. Since the action of Cpx on membranes is stoichiometric, rather than catalytic, we observe that Cpx exerts dramatic effects upon initial membrane binding, but then appears to eventually reach a steady state.

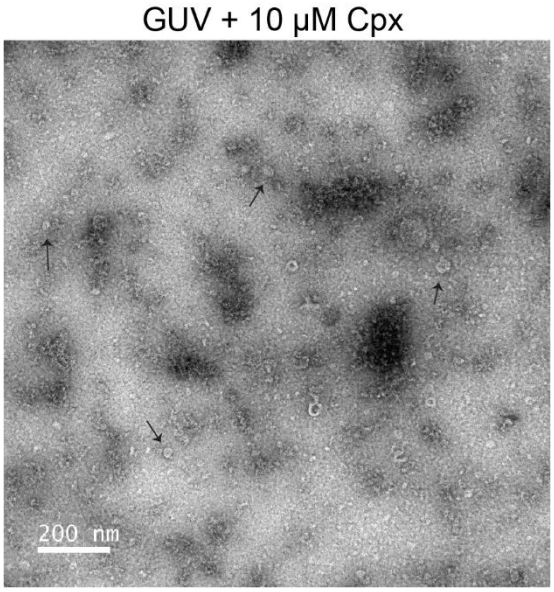

Fig. R7 Negative stain EM of GUVs after treatment with 10 µM Cpx.

d. Minor: The kinetics of the of the fluorescence increase could be better analyzed in terms of diffusion. What is the gyration radius of JF635i? What is the concentration in bulk of JF635i? How can these gyration radius and concentration explain such a fast increase of fluorescence in 10 s? Is it quantitatively consistent with the diffusion of the dye?

Author response: The structure of the JF635i dye is shown in Extended data Fig.S1c. At present, an accurate radius of gyration is unknown for this specific HaloTag ligand. As a reasonable approximation, we used Pymol and RCSB PDB 6U2M to calculate the radius of gyration from a comparable HaloTag ligand, JF635 (a membrane permeant variant); the radius of gyration for JF635 was determined to be 6.63 Å. Using this value, the diffusion coefficient was calculated to be 2.6 x 10^2 µm^2·s^{-1}, according to the following equation from He and Niemeyer, where T is temperature in Kelvin and η is viscosity (2008, https://doi.org/10.1021/bp0256059):

$$D = 5.78\ x\ 10^{-8}\left(\frac{T}{\eta\ \mathrm{x}\ \mathrm{Rg}}\right)$$

When considering this value, we do not expect diffusion to be rate limiting in this reaction. The reaction is also governed by the kinetics of pore formation, dye permeation through the pore and the HaloTag binding and reaction kinetics. While the rate of dye permeation is unknown for these particular experiments (but was previously demonstrated to be a rate-limiting component[8]), the MD simulations demonstrate that pore formation can take place within 500 ns and we expect Cpx to generate multiple pores in the GUVs to facilitate rapid entry/exit through the membranes. The JF635i binding kinetics are also sufficiently rapid (k_{on} = 0.87 ± 0.1 μM^{-1} s^{-1}) [9] to justify the observed fluorescence changes in our GUV imaging. In particular, our GUV experiments were performed with 2 µM JF635i in the media and the GUVs were generated in the presence of 10 µM HaloTag protein; the external protein was washed away, leaving only encapsulated protein. Assuming a GUV has an average diameter of 20 µm, with an inner volume of approximately 4 femtoliters, only 0.4 zmoles of HaloTag molecules are contained within each GUV. So, we expect the HaloTag protein to become rapidly saturated with ligand after pore formation.

2. Pore forming activity of Cpx

The experiments and the simulations form a very convincing set of data. There may be some ways to make the results a bit more quantitative.

a. Can the authors estimate an equivalent Cpx concentration in the simulations and compare it with the experiments?

Author response: The MD simulations for this study were performed using a bilayer composed of 130 phospholipids (DOPC/DOPS, 104:26). In total, we simulated the interaction of 4, 6, 9, 10, 11 and 12 Cpx Ct21 peptides with the phospholipid bilayer, corresponding to a peptide/phospholipid ratio ranging from 1:30 to 1:11. Due to the small dimensions of the simulations box (9.0 × 9.0 × 11.5 nm^3), the concentration range in this system is calculated to range from 7.5 mM to 21 mM (note that the dimensions of this system are typical for atomistic MD simulations). In contrast, the Cpx concentrations used in the various experiments were 50

nM to 10 µM. We therefore do not think that the relative concentrations can be compared between these two systems.

b. Why does the Cpx pore reseal? Is it due to a low resealing energy barrier or to the removal of one or more peptides? This may be discussed in view of the lifetime distributions at various concentrations (below 500 nM).

Author response: When applying a low concentration of Cpx (50 nM) into the *cis* chamber of the BLM system, we observed the opening of transient pores in the phospholipid bilayer. These opening events occurred in low frequency and were short-lived; so, they could only be studied qualitatively in our study. Upon increasing the Cpx concentration to 500 nM we observed stable single pores that remained mostly in the open state for tens of minutes; these stable single pores were quantitatively analyzed. We believe the differences between transient and stable Cpx pores in the BLM are attributed to differences in the 'local copy number' of the Cpx molecules in the two conditions. A low concentration of Cpx would result in a reduced probability for 12 Cpx molecules to find each other along the BLM surface and assemble into a stable pore (based on the MD simulations). Therefore, when the local copy number is low, Cpx will have a limited capacity to dilate the pore, and thus the energetics of the system would favor the resealing of a pore into the closed state. Interestingly, our MD simulations data shows that once a pore has formed, as few as four Cpx peptides are sufficient to stabilize an open pore for hundreds of nanoseconds (Fig. 2g). Although outside the scope of our current study, it would certainly be insightful to use MD simulations to explicitly analyze and quantify the magnitude of the energy barriers governing the opening and closing events in the low copy number conditions.

In light of this reviewer comment, we revisited our 500 nM Cpx data to extract additional information from the open and closed dwell time distributions from the stable Cpx pores. Similar to our previous work [10], we generated a cumulative distribution function from the open and closed dwell time distributions, and fitted them with exponential functions. From this analysis, we calculated the open rate and closed rate of the pores, which were then used to derive the Gibbs free energy change from the closed state to the open state. The open and close rate

values were determined to be: 0.04 and 2.26 (1/ms), respectively. We have now included this information in the revised manuscript and thank the reviewer for prompting this additional analysis.

c. SUMO-Ct21 displays transient pores of smaller size than Cpx at 500 nM. What could explain this behavior?

Author response: Dr. Pincet correctly points out that full-length Cpx generated pores in the BLM that were larger and more stable than SUMO-Ct21. We observed that the full-length protein generated consistent pores, while SUMO-Ct21 gave rise to pores that were more variable. We believe that the full-length protein and the SUMO-Ct21 construct will interact in distinct ways with phospholipid bilayers. Specifically, we expect that the orientation of the inserted Ct21 helix would be more regularly organized by the full-length protein, while the SUMO-Ct21 could exhibit greater lateral and rotational mobility and thus result in a more disorganized pore. Alternatively, the SUMO domain on the SUMO-Ct21 protein could sterically influence how the Ct21 helix associates with the phospholipid bilayer. It is also possible that other residues (not within the Ct21 helix) could engage in homomeric interactions upon membrane binding, that serve to organize and stabilize the pore.

d. When zooming in around the transient pores, is a plateau observed? If so, fluctuations around the plateau value would provide direct information on the energetics of the pore.

Author response: As described above, the transient Cpx pores that we observed in the BLM were short-lived openings (a few seconds or less) that occasionally appeared throughout an otherwise quiet recording. These pores produce only a few fluctuating data points. In order to accurately analyze pore dynamics in the BLM system, a single pore must remain active for at least several minutes in order to accumulate sufficient data. As such, we are unable to quantify the energetics of these transient pores. Bearing in mind that we do not believe Cpx is a pore-forming molecule in mammalian cells (but instead acts as a pore stabilizing agent), we found that stable pores required a higher concentration of Cpx to facilitate pore assembly. Based on

the MD simulations, the transient Cpx pores likely represent openings in the BLM that are formed by greater than eight, but fewer than twelve molecules.

e. The HEK patch-clamp experiments (pore formation in the plasma membrane) are not fully conclusive. How do the authors explain that in 14 out of 21 trials there was no event observed with WT Cpx?

Author response: When performing cell-attached patch electrophysiology, we found that Cpx in the pipette solution caused positive current to flow through the HEK cell plasma membrane in 7 out of 21 trials. We believe this movement of positive current represents pore formation in the plasma membrane. We speculate that the lack of an observed event in the negative trials is due to interferences in the ability of Cpx to assemble in sufficient local numbers to generate a pore. For example, the outer surface of the mammalian cell plasma membrane is heavily glycosylated and densely packed with protein. When considering our CaM competition experiments (Extended data fig. S5), we believe the outer surface of the cells contain (potentially numerous) non-canonical Cpx binding partners that would reduce the pore formation efficiency. In addition to this likely scenario, it is also possible that Cpx can bind the surface of the glass patch pipette, thus lowering the effective concentration. We used 10 µM Cpx in these experiments, as this was sufficient for our other assays. Pore formation would likely be more efficient by increasing the concentration. However, regardless of the imperfect result with 10 µM Cpx, we did not observe any currents in any of our control conditions, including the ΔCt21 Cpx condition. We are therefore confident in our conclusion that Cpx can form plasma membrane pores. We have now directly acknowledged the inefficiency of plasma membrane pore formation in the revised manuscript as follows:
“The limited success rate for pore formation in these experiments might be due to the presence of inhibitory factors or non-canonical interactions on the crowded surface of cells.”

f. Regulation of the pore forming activity by phosphorylation and Calmodulin. Because of the issues on the GUV experiments, experiments at 1µM Cpx or less would be more appropriate,

or, even better, electric measurements just like Fig. 1. Also, it is not clear what the white pixels represent in the GUVs (Fig. S5b)

Author response: As described above, our GUV experiments were performed without glycerol in the solutions. We trust that our response above should alleviate concerns about the concentration of protein used in Extended data Fig. S5. The purpose of Extended data Fig.S5 is to provide proof-of-principle that Cpx membrane remodeling activity is likely to be regulated *in vivo*. We identify phosphorylation and protein-protein interactions as two possible mechanisms that block or reduce Cpx membrane remodeling activity. We feel the current iteration of this figure effectively demonstrates this.

Regarding the white pixels in Extended data Fig. S5b, the GUVs were labeled with 0.1 % rhodamine-DOPE. The rhodamine fluorescence was pseudo-colored in white. Although this information is written in the Materials and Methods section, we appreciate this clarifying comment and we have now also included this detail in the figure legend.

g. Minor. It would be helpful to show (draw) the connection between the pore itself (Fig. 2b, 12 peptides) and the various diameters presented in Fig 2.e. It is hard to see the 7 nm when 1nm away from the minimum pore diameter. For instance, a few arrows showing the various diameters on one of the pictures may be a good solution.

Author response: We thank the reviewer for this suggestion, as the original version of Fig. 2e was somewhat difficult to interpret. To make this panel of data unambiguous, we have rotated the graph 90° and included an inset showing a MD simulations snapshot. We feel the revised version of Fig. 2e, shown below, is far more intuitive than the original version, and will be clearly interpretable for readers.

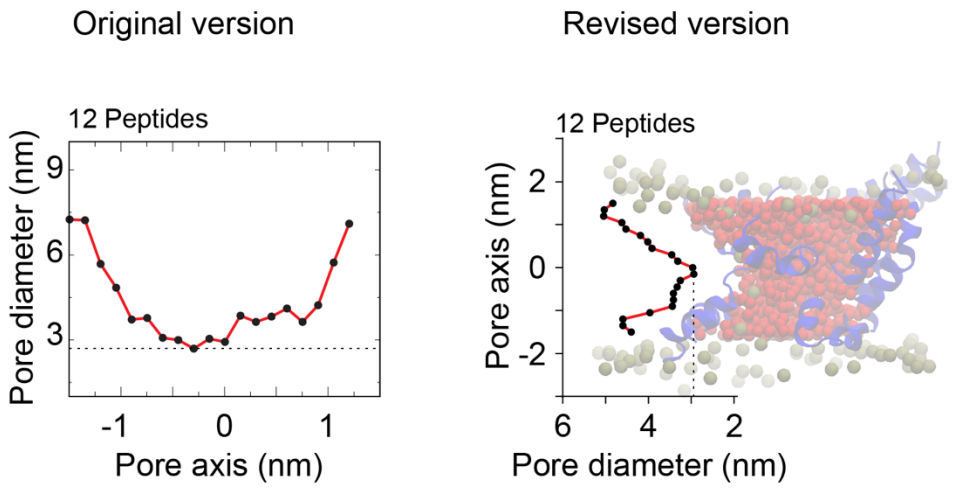

Fig. R8 – Comparison of the original and updated version of Fig. 2e.

Please also note that the values at the outer edges of the pore reported in the two plots are different. The diameters at two ends of the pore are much larger than at the center because the pore is a distorted toroid, rather than a cylinder. While water molecules in the central part of the pore can be clearly identified, water molecules inside and outside the pore are difficult to distinguish at the membrane surface. In the previous pore-axis vs pore-diameter plot (Fig. R4, *left panel*), some water molecules that are not part of the pore were included in the calculations of diameters near the membrane interface. We have corrected this in the current revision. We note that Fig. 2f and 2g remain unchanged, as these are based on the unambiguous central part of the pore; Fig. 2g is calculated by taking the average of 1000 frames.

3. Stabilization of the SNARE-induced fusion pore. This part is nicely done; no comment.

4. Minor. Please, carefully proofread the manuscript. Here are a few typos/missing details but there are probably many more:

a. Line 120 Fig 3a not 1a

b. Line 121 and 124: Fig 3 not 1

c. Fig. S5e, no need for x 10^3 for an arbitrary unit.

Mat. and Met.

d. Lines 12, 17 and 24 ° has to be indicated instead of the current symbol

e. Line 35: What chains (PC and PS not sufficient)?

f. Line 48: Fig. 3

g. Line 59: For extrusion it is always an odd number to avoid recovering the initial aggregates. Is it really 50?

h. Line 220: is it really 5µm?

i. ND-Black Lipid membrane description is confusing. Why start with SNAREs when it is only used at the very end?

Author response: We are grateful for Dr. Pincet's keen eye for detail. We have corrected or modified all of the above suggestions from Specific comment #4 accordingly.

Frédéric Pincet

1. Bao, H. et al. Dynamics and number of trans-SNARE complexes determine nascent fusion pore properties. *Nature* **554**, 260-263 (2018).
2. Sun, D.L., Forsman, J. & Woodward, C.E. Molecular Simulations of Melittin-Induced Membrane Pores. *Journal of Physical Chemistry B* **121**, 10209-10214 (2017).
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4. Malsam, J. et al. The carboxy-terminal domain of complexin I stimulates liposome fusion. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 2001-2006 (2009).
5. Seiler, F., Malsam, J., Krause, J.M. & Sollner, T.H. A role of complexin-lipid interactions in membrane fusion. *Febs Letters* **583**, 2343-2348 (2009).
6. Ramakrishnan, S., Bera, M., Coleman, J., Rothman, J.E. & Krishnakumar, S.S. Synergistic roles of synaptotagmin-1 and complexin in calcium-regulated neuronal exocytosis. *Elife* **9**, 18 (2020).
7. Das, D., Bao, H., Courtney, K.C., Wu, L. & Chapman, E.R. Resolving kinetic intermediates during the regulated assembly and disassembly of fusion pores. *Nat Commun* **11**, 231 (2020).

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9. Jonker, C.T.H. et al. Accurate measurement of fast endocytic recycling kinetics in real time. *J Cell Sci* **133**(2020).
10. Wu, L., Courtney, K.C. & Chapman, E.R. Cholesterol stabilizes recombinant exocytic fusion pores by altering membrane bending rigidity. *Biophys J* **120**, 1367-1377 (2021).

Decision Letter, first revision:

23rd Nov 2021

Dear Ed,

Thank you for submitting your revised manuscript "The complexin C-terminal amphipathic helix stabilizes the fusion pore open state by sculpting membranes" (NSMB-A45178A). It has now been seen by two of the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Structural & Molecular Biology, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines. I would like to note that we were aware of the work mentioned by reviewer #3 and therefore do not share their concerns about the novelty of the findings of this study. You may still consider textual changes to make the advance clearer.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Structural & Molecular Biology Please do not hesitate to contact me if you have any questions.

Kind regards,
Florian

Florian Ullrich, Ph.D.
Associate Editor
Nature Structural & Molecular Biology
ORCID 0000-0002-1153-2040

Reviewer #1 (Remarks to the Author):

I am impressed by the new data. The authors have substantially bolstered their observations regarding the pore-stabilizing role of the Cpx C-terminal domain and convincingly dealt with my concerns throughout the edited manuscript. I could not find the Methods section in this revised document, so I was not able to evaluate everything in this read through, but I don't recall major issues in that section of the previous draft – just a few typos like 5 microns when perhaps you meant 5 nanometers. Another reviewer pointed that typo out previously I believe.

I was struck by the new ND3 data and the observation that the pore opening rate was significantly enhanced when Cpx was included (Fig. 5G). In contrast, the pore opening rate was already at the larger value when 5 vSNAREs were present and Cpx had no further effect. It's as if both SNARE copy number and Cpx-CTD can lower the energy barrier for fusion pore formation to some extent, but that some other energy barrier then becomes rate-limiting and sets the upper limit for the observed open rates. There was little if any mention of this in the Results and nothing in the Discussion - I found this to be a fascinating observation. I recognize that detailed discussions of the energy barriers for fusion and pore formation are not warranted here, but the data make a strong case for the CTD of Cpx having the capability of lowering the energy barrier for pore opening and compensating to some extent for fewer SNAREs. It seems like yet another previously unappreciated aspect of the Cpx CTD and one that fits nicely with the AMP model conceived in this study. This is only a suggestion and I leave it to the authors' discretion. Overall, this is a fascinating and expertly crafted study, and the manuscript was a pleasure to read.

Reviewer #3 (Remarks to the Author):

I am happy with most of the author's response to the reviewer comments. I am relieved that there was no glycerol in the GUV but am still not fully convinced that the GUV results and EM pictures are really consistent. In any case, this is what is observed and it does not change the conclusions of the study. However, I still have a couple of concerns.

Major concern
In the paper by Malsam et al, Cell Reports 32, 107926, July 21, 2020 (that is not cited in the submitted manuscript), it is indicated that "The C-terminal region contains a short amphipathic helix that binds high-curvature membranes and modulates species-specific inhibition" and they provide several references (Gong et al., 2016; Seiler et al., 2009; Snead et al., 2014; Wragg et al., 2013,2017; Zdanowicz

et al., 2017). This is very reminiscent of the conclusions presented here and casts doubt on the novelty of the present manuscript. In view of this, the authors absolutely need to better discuss the novelty of their results. I do apologize for not mentioning it before but I only discovered this paper after my initial review.

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The uploaded Word version of the manuscript is not the final one (there are still personal comments from the authors). Is the revised manuscript (pdf and/or Word) exactly the same when accepting the changes? If not, I would have liked to see the final version.

Frédéric Pincet

Author Rebuttal, first revision:

Response to reviewer comments

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Cpx CTD and one that fits nicely with the AMP model conceived in this study. This is only a suggestion and I leave it to the authors' discretion. Overall, this is a fascinating and expertly crafted study, and the manuscript was a pleasure to read.

Author response: We are pleased to learn that the reviewer was impressed by the new data that was included after revision. Indeed, the new ND3 results, inspired by the reviewer, are a great addition to our study and we, again, thank the reviewer for this suggestion. We also appreciate the above suggestion to describe how Cpx affects the energy barrier(s) for fusion. We have now elaborated on this in both the Results and Discussion sections of the updated manuscript and included three excerpts below. The new text is displayed in green.

Results section 1- page 7

"In line with our previous work[20, 21], NDs with three copies of syb2 (ND3) formed small and transient pores with a current of approximately 1-2 pA, and an open time fraction of ~0.1 (Fig. 5d and 5e). The instability of ND3 pores make this condition especially sensitive to factors that affect their kinetic properties. Strikingly, incorporating three TMD-Cpx molecules into ND3 greatly increased the size of fusion pores, as evidenced by an increase in the average current to 6 pA (Fig. 5e, *left panel*). Under these conditions, TMD-Cpx also dramatically stabilized fusion pores in the open state, yielding an open time fraction of nearly 1.0 (Fig. 5e, *right panel*). Moreover, TMD-Cpx caused a significant shift in the open dwell time distribution, to >100-fold longer open times (Fig. 5f), and the closed dwell time distribution was shortened by 10-fold. Further kinetic analysis revealed that the opening and closing rates of pores with TMD-Cpx were significantly increased and decreased, respectively, as compared to the syb2 alone control (Fig. 5g). These findings indicate that TMD-Cpx has two effects on ND3 fusion pores, it lowers the energy barrier for opening, and increases the barrier for closing."

Results section 2 – page 8

"Although the ND3 studies are particularly useful for examining factors that stabilize nascent fusion pores, this condition is less well suited to study inhibitory factors. In the next series of experiments, we assessed the effect of Cpx on already-stable pores by increasing the copy number of syb2 to five (ND5). To maintain a 1:1 stoichiometry, TMD-Cpx was co-reconstituted at the same copy number. Under these conditions, ND5 alone yielded stable pores with large currents (Fig. 6), as previously reported[20, 40]. In contrast to ND3, incorporation of TMD-Cpx did not affect the current, open time fraction, or the closed dwell-time distribution (Fig. 6a-c) of ND5 pores, as compared to control. However, the peak of the open dwell-time distribution exhibited a large, 10-fold shift to the right (Fig. 6c). Further analysis revealed that the closing rate of ND5 TMD-Cpx pores was significantly slower (eight-fold) than the control (Fig. 6d). Hence, for both ND3 and ND5, TMD-Cpx stabilizes the open state of SNARE-mediated fusion pores. However, TMD-Cpx exerts a more dramatic effect when acting upon pores formed by a smaller number of SNAREs. In particular, TMD-Cpx affected both the opening and closing energy barriers of ND3 pores, while for ND5 pores, only the energy barrier for closing was affected. Interestingly, increasing either Syb2 or Cpx copy number (starting with the ND3 condition) had similar effects on these energy barriers, suggesting Cpx may compensate when the SNARE copy number is limited."

Discussion section – page 11

"To restrict the pore forming activity of Cpx in our ND-BLM system, we tethered Cpx to syb2-containing NDs using the TMD of CD4. This strategy prevented the formation of spurious Cpx pores in the bilayer (Extended Data Fig. 6) and facilitated 1:1 stoichiometric control of Cpx with *trans*-SNARE complexes. When Cpx was specifically localized to the nascent fusion pore site, it significantly stabilized the open state in both ND3 and ND5 conditions (Fig. 5 and 6), with a greater stabilizing effect on ND3 pores. We propose that this stabilization effect is due to changes in the energy barrier for fusion via membrane insertion of the Cpx C-terminal amphipathic helix, because: 1) the isolated Ct21 peptide has the intrinsic ability to form pores and remodel bilayers (Fig. 1 and 2), 2) this helix can be substituted for an alternative amphipathic helix, metlittin, with rescued function (Fig. 5), and 3) C-terminal truncation significantly destabilizes the fusion pore open state (Fig. 5 and 6). Indeed, truncation of the C-terminal amphipathic helix eliminated the fusion pore stabilizing activity of TMD-Cpx with ND3 (Fig. 5) and acted as a strong inhibitor with ND5 (Fig. 6)."

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Frédéric Pincet

Author response: We are pleased that the reviewer was happy with our revised manuscript. For clarification, the revised manuscript that we supplied was indeed the most up-to-date 'tracked-changes' version. The 'accepted changes' version was otherwise identical.

We appreciate the comments that the reviewer raised about the Malsam (2020) Cell Reports study. The references included within that study cover several reports that show the C-terminus of Cpx binds membranes. We were aware of these studies and, in fact, also included 5 out of 6 of these references in our manuscript. Although these referenced studies describe Cpx-membrane interactions, they do not report that Cpx exhibits membrane deformation or pore formation activity. This is a major distinction between our work and the existing literature.

While we are aware of the Malsam et al (2020) study, we did not include it for a number of reasons. For example, their study claimed that Cpx is a fusion clamp (as indicated in their title), but the triple KO data clearly show it is not a clamp (Fig. 4B in their study). Moreover, their study (and the Malsam et al 2012, EMBO study cited in our manuscript) included EM on GUVs that were incubated with SUVs in the presence of Cpx. Neither of these Malsam studies report any imaging of a complexin-free sample. Without this crucial control, it cannot be determined if the authors are reporting docked SUVs, or Cpx-induced GUV vesiculation. So, we think that citing this study might add confusion; hence, we omitted it. Although we prefer to keep our reference list as-is, we can include a reference to Malsam (2020) in our manuscript, if deemed absolutely necessary by the reviewer or editor.

Final Decision Letter:

14th Dec 2021

Dear Ed,

We are now happy to accept your revised paper "The complexin C-terminal amphipathic helix stabilizes the fusion pore open state by sculpting membranes" for publication as a Article in Nature Structural & Molecular Biology.

Acceptance is conditional on the manuscript's not being published elsewhere and on there being no announcement of this work to the newspapers, magazines, radio or television until the publication date in Nature Structural & Molecular Biology.

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Kind regards,
Florian

Florian Ullrich, Ph.D.
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