Distinct proteostasis states drive pharmacologic chaperone susceptibility for Cystic Fibrosis Transmembrane Conductance Regulator misfolding mutants

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1st Editorial Decision

RE: Manuscript #E21-11-0578
TITLE: “Distinct proteostasis states drive pharmacologic chaperone susceptibility for Cystic Fibrosis Transmembrane Conductance Regulator misfolding mutants”

Dear Dr. Plate:

Thank you for your robust response to the Review Commons critiques. I agree that your revised submission, combined with the proposed additional experiments in the revision plan satisfactorily address the most pertinent concerns. We would therefore be happy to receive a fully revised version that contains the additional new experiments described in the revision plan.

Sincerely,
Elizabeth Miller
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Plate,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) 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).

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Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available

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Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,
Eric Baker
Journal Production Manager
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Response to Reviewer Comments

Reviewer 1, general comments: In this manuscript, the authors used multiplexed AP-MS to compare the interactomes of different Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) variants with or without a corrector drug, VX-809. They included four variants, the most common F508del variant which shows a mild response to the drug (hypo-responder), two hyper-responders P67L and L206W, and another hypo-responder variant G85E. They showed that the TMT-based quantitative proteomics results recapitulate the previously reported interactome data from Pankow et al. (2015) and Hutt et al. (2018). They further found the global protein-protein interactions for two hyper-responders shifted to near WT level due to VX-809. In addition, they propose that VX-809 may bind to CFTR hyper-responders before the proteasomal degradation step of the CFTR biogenesis. Finally, they investigate the impact of the degradation pathways - ERAD and autophagy - on the VX-809 correction.

This work not only shows that the TMT-based quantitative proteomics approach is successful at reproducing the interactome data of CFTR - both WT and variants, reported in the past, but also provided the interactome data of two other CFTR variants (P67L and L206W) with or without VX-809 and G85E with VX-809 for the first time. Moreover, and most interestingly, this work demonstrated that the mechanistic basis of different theratypes can be correlated with differences in the CFTR interactome. Overall, the experiments are well designed, the technical aspects of the work are sound, and the results are very interesting. There are a few suggestions, below.

Reviewer 1 (Significance): The work will be of strong interest to researchers interested in CFTR folding/misfolding and proteostasis correction, as it yields some intriguing mechanistic hypotheses that could likely influence future therapeutics development. More broadly, the work will be valuable to the proteostasis, membrane protein biology, and proteomics communities.

Our response: We thank reviewer 1 for this positive and constructive assessment, as well as highlighting the appeal to a broad audience. We addressed all concerns in detail below.

Reviewer 1, comment 1: The paper is tough to follow, especially the sections about, for example, proteasome inhibitors attenuating the VX-809 response.

Our response: Thank you for this feedback. We rewrote the section on the proteasome inhibitors significantly to reduce some details and provide more clarity. We focused on the essential observations: 1) The correction of P67L trafficking is attenuated by proteasomal inhibition and 2) F508del trafficking correction is unaffected by proteasome inhibition. Furthermore, we streamlined Figure 6 to contain only the former panels A, C, D, F, G, and H, and we moved panels B, E, I and J to the Supplemental Materials section (Supplemental Figure S9) to reflect the reduction in complexity in the proteasome inhibitor section.

Reviewer 1, comment 2: On line 277-280 page 10 and line 498 page 17, the authors claim that the interactome of P67L+VX-809 resembles that of WT more than P67L with DMSO. However, the supporting evidence for this claim is Supplemental Figure S4B, which shows that there are three more overlapping interactors between P67L+VX-809 and WT than that of P67L+DMSO and WT. Three is not a large number to base such a claim. Points that derive from that claim should be modified.
**Our response:** We agree with Reviewer 1. We removed the conclusions in line 281 claiming “Thus, P67L treated with VX-809 resembles WT interactome more than P67L with DMSO.” For line 498, see Reviewer 3 minor comment 6, where Reviewer 2 asked us to change the word “resembles” to “shifted towards”. We clarified that this claim is derived not from overlap, but from the heatmaps which show a decrease in interaction levels for P67L + VX-809 when normalized to WT. The text now reads as follows:

“In the presence of VX-809, the P67L interactions shifted towards WT levels, as demonstrated by P67L + VX-809 in our heatmaps (Figure 4B).”

**Reviewer 1, comment 3:** Line 445-446 page 16, “In contrast, E-64 abrogated the small degree of VX-809 correction for F508del.”: I don’t think I see clear evidence of this. The bar graphs do not show the statistical tests for this purpose.

**Our response:** We thank reviewer 1 for pointing out this discrepancy. We have now removed any reference to the E-64 inhibitor modulating the VX-809 response for F508del. Instead, we conclude that a moderate restoration of band C F508del can take place in the presence of E-64 as reflected in Figure 6E-F. As discussed above (reviewer 1 comment 1), we significantly revised the text in the section. We specifically address this point:

“Similarly, E-64 did not impact the modest F508del correction afforded by VX-809 (Figure 6E-F, Supplemental Figure S9A-B).”

**Reviewer 1, comment 4:** line 161 page 6, “93 and 73 interactors”: 93 should be 83 according to Figure 2B.

**Our response:** We corrected this typo.

**Reviewer 1, comment 5:** line 194 page 7, “Figure 1C”: I think Figure 1B is more relevant here.

**Our response:** We agree with Reviewer 1, we changed this reference to Figure 1B.

**Reviewer 1, comment 6:** line 218 and 219 page 8, “Hutt et al.” & “Pankow et al.”: Please be consistent with comma.

**Our response:** We added a comma after Hutt, et al. and Pankow, et al. on line 218 to be consistent.

**Reviewer 1, comment 7:** line 354 page13, “27 G85E interactors overlapped ~”: Figure S5E actually shows that there are 32 overlapping interactors, and I assume if this is not a typo, it stems from including the green dots (the interactors that enriched but did not pass the statistical significance). If so, I think the authors should be consistent here with Figure 2B and include only the interactors that pass the statistics test in the Venn diagram (Supplemental Figure S5E) to prevent confusion.
Our response: We thank Reviewer 1 for pointing out this error. The correct number of overlapping interactors is 32, not 27. We were consistent with not including the green dot interactors in all the overlap plots in this paper.

Reviewer 1, comment 8: line 389 and 390 page 14, please change both of the in-text figure references to the relevant ones.

Our response: We changed the reference from Figure 4D to Figure 4C and the corresponding reference from Figure 5E to Figure 5D to reference the correct panel.

Reviewer 1, comment 9: line 407-408 page 14, typo on "proteome"

Our response: We corrected this typo.

Reviewer 1, comment 10: line 446 page 16, in-text figure reference missing 6 in front of G-H

Our response: We added the number 6 to reference Figure 6G-H (please note that these have been renumbered and some panels moved to Supplemental Figure S9).

Reviewer 1, comment 11: line 482-483 page 17, "Importantly, our TMT-based quantification provides the ability to directly compare F508del interactions under VX-809 treatment." Although it was label-free, the interactome study with or without VX-809 on F508del variant was already performed in Hutt et al. (2018), and thus it should be acknowledged by either mentioning in text or by citation.

Our response: We apologize that this point was not clear. The introduction references the prior study by Hutt, et al.:

“VX-809 treatment also modulates PN interactions and remolds the F508del interactome towards WT37.”

We also edited the text in the discussion to again acknowledge the previous study as follows:

“Importantly, our TMT-based quantification provides the ability to directly compare F508del interactions under VX-809 treatment, and although previous studies have made similar comparisons, they used label free quantification.”

Reviewer 1, comment 12: line 531 page 18, "Supplemental Figure 4B": should be Figure 4B.

Our response: In this context we are not discussing the grouped protein interaction data from Figure 4B, but we are specifically discussing DNAJB12, which is downregulated by VX-809 in the correlation plot in Supplemental Figure S4F – right panel. To clarify this point we made the following alterations to the text:

“Indeed, we see that P67L-DNAJB12 interactions are particularly reduced among folding factors by VX-809 when correlating the interaction fold changes (Supplemental Figure
Reviewer 1, comment 13: line 657 page 22, "splice-isoforms and supplemented": should be a space between "isoforms" and "and".

Our response: We added a space between these words in the text.

Reviewer 1, comment 14: line 674 page 23, "a final concentration of 3nM": should be 3uM.

Our response: We corrected this typo.

Reviewer 1, comment 15: line 918 page 32, "IP elution are shown at the bottom": missing period at the end.

Our response: We added this period.

Reviewer 1, comment 16: line 922-923 page 32, "Duplicates of P67L and F508del treated samples with tdTomato (mock), and WT controls were purified via IP": this sentence needs to be edited to prevent confusion.

Our response: Thank you for pointing this out. We modified the text as follows:

“VX-809 treated and untreated samples of WT, P67L, F508del, L206W, and G85E CFTR, as well as a tdTomato (mock) control were purified via IP.”

Reviewer 1, comment 17: The authors should mention that the differences between the interactome data in this paper and the data from previous papers (Hutt et al. and Pankow et al.) may partially come from the use of different cell lines.

Our response: We added this detail. Specifically, we added the following sentence in the section comparing our interactors overlap:

“Some difference in identified interactors may be attributed to the use of different cells lines between our study and previous work.”

Reviewer 1, comment 18: The authors did not provide a direct comparison between P67L and L206W interactome data. Although they both belong to the same theratype, I think it would be interesting to note the similarities and differences of interactors between two variants, which may be resulting from the location and unique characteristics of the mutations within the CFTR protein structure.

Our response: We agree with Reviewer 1 that comparison of P67L and L206W would be interesting. We plotted the protein quantification correlation plots of six pathways discussed at length in the text: translation, folding, proteasomal degradation, autophagy, trafficking, and endocytosis. We included these plots in an additional Supplemental Figure S7. Notably, we
found that correlation between the two hyper-responders increases for later pathways e.g. trafficking and endocytosis and call attention to this point in the text:

“P67L and L206W interactors correlate moderately for proteins early in biogenesis such as translation, folding, and degradation, and correlation increases for later pathways such as trafficking and endocytosis regardless of DMSO or VX-809 treatment (Supplemental Figure S7A-F).”

Reviewer 1, comment 19: Figure 1A: In the structure, there are two residues that are color coded with either yellow or purple. They should not be there. Also, there is a typo on P67L description: “Rescued functio...” -> function:

Our response: The structure in Figure 1A was updated to only highlight the relevant residues in colors: blue (P67L), green (L206W), orange (F508), and red (G85E). Furthermore, the typo was corrected to “function”.

Reviewer 1, comment 20: Figure 1B: Under “Experimental IP”, where it says "WT, F508del, or P67L CFTR", I think the authors should include other variants or at least say "other variants" or "etc". Also, the chemical structure of VX-809 should be on the left side, not on the right side of the scheme. Its current position is kind of random and does not carry any meaning.

Our response: Thank you for this feedback about Figure 1. We have significantly revised Figure 1. We added “etc.” under “Experimental IP” to clarify that we are performing this experiment for multiple mutations. Additionally, we moved the VX-809 structure to the left side of the diagram and wrote “add VX-809” to clarify the purpose of showing the structure in the experimental schematic.

Reviewer 1, comment 21: Figure 1C: In the table above the western blot data, I think it is better to write "VX-809" instead of "Lum.". That way, it will be more straightforward to the readers and also consistent with other figures.

Our response: As discussed above, we have made important changes to Figure 1 to increase clarity. We replaced “Lum” with “VX-809” in this context.

Reviewer 1, comment 22: Figure 1D: I am assuming that the small bar graph on the right bottom corner to explain the overall TMT-based MS/MS is an example and does not represent any data from this paper, but since it uses the same color codes with the actual bar graph on the left bottom of this figure, it would be better to be consistent in the relative size of each bar between two graphs.

Our response: We altered the small bar graph in Figure 1D to reflect the relative size of quantification from the large bar graph in Figure 1D. Furthermore, we increased the size of the peptide quantification plot and removed details on the difference between experimental and theoretical peptide sequence.

Reviewer 1, comment 23: Figure 7: The chemical structure of VX-809 should be correctly
drawn. Perhaps the model would be a better representation of the data if the authors show less of interacting proteins in Figure 7B compared to Figure 7A.

**Our response:** We agree with Reviewer 1 and replaced the VX-809 structure in Figure 7 with the correct detailed structure. We also thank Reviewer 1 for the suggestion to reduce the interacting proteins in Figure 7B to reflect our observation for the hyper-responsive mutants. We removed several of the drawn interactors (ribosome, proteasome, several interacting chaperone proteins), as well as reducing the size of the autophagosome depicted in 7B.

**Reviewer 2**

**Reviewer 2, general comments:** The manuscript submitted by Plate and colleagues continues their exploration of altered protein-protein interactions in various disease states, with the aim of identifying novel potential therapeutic avenues. In this study, the authors detect and quantify the interaction of cellular quality control machinery with WT CFTR protein as well as four different disease-causing CFTR mutants, all expressed in HEK293T cells, and treated with either the FDA-approved CFTR corrector Lumacaftor (VX-809) or a vehicle control. They chose CFTR mutants which are all categorized as Class II mutations, exhibiting altered protein folding and trafficking and with decreased localization to the cell surface. However, two of the mutants (P67L and L206W) are highly "correctable" by VX-809 ("hyper-responders"), while the other two are mildly (F508del) or completely (G85E) resistant to correction ("hypo-responders"). Through co-immunoprecipitation coupled to tandem mass tag multiplexed LC-MS/MS, the authors identified the interactome for each CFTR variant under both treatment conditions and quantified the relative abundance of each interacting peptide. The authors found that, as expected, the proteostatic network differed between WT CFTR and the disease-causing mutants. Specifically, each mutant had significantly increased interactions with cellular quality control machinery in several key pathways, including translation, translocation, folding, trafficking, membrane organization, and proteasomal and autophagic degradation. Interestingly, the CFTR mutants displayed variable changes in proteostatic factor interactions with upon treatment with VX-809, and these effects mirrored correction. While the interactome of the two hyper-responders (P67L and L206W) shifted toward more WT-like levels, the abundance of interactions of the hypo-responders either were not as reduced (F508del) or unaffected (G85E). The authors next compared the abundance of interactions with individual proteostasis pathways, which they grouped by GO-terms and organized in the order of biogenesis. They found that while P67L, L206W and F508del all had similar reduced interactions with factors that participate in early biogenesis (translation and folding), F508del interactions with later quality control machinery (proteasomal and autophagic degradation) were not reduced to the same extent that was seen for the hyper-responders. They suggest that VX-809 changes proteostatic pathway interactions at an inflection point between folding and degradation for the more correctable mutants, which is not surprising based on prior work from other labs. The authors also posited that inhibiting the proteasome or lysosome might rescue maturation of the CFTR variant proteins to more closely resemble WT. However, they observed by western blot that while immature (A/B band) CFTR rose for the hyper-responders upon bortezomib addition, when VX-809 is also added maturation to band C is instead attenuated. Inhibition of lysosomal degradation by E-64 had no effect on hyper-responders' correction by VX-809. Immature G85E CFTR also increased upon proteasome addition, but no maturation to C band was observed in any of the tested conditions. In contrast, proteasome inhibition resulted in increased B and C bands for F508del CFTR, and more maturation to C band occurred when bortezomib and VX-809 were combined relative to when either was added alone. Again, in contrast, treatment with E-64 attenuated maturation of F508del CFTR to band C when VX-809 was also added. The authors therefore suggest a role
for altered protein degradation as a result VX-809 correction, which is line with rescue.

Overall, this work expands on two previously published proteomic studies that examined the CFTR proteostatic interaction network. Indeed, the authors compared their results to these studies, and while the individual components they identified as interacting with CFTR do not overlap to a high degree—indicating the discovery of some novel interactions—the proteostatic pathways involved in CFTR biogenesis are similarly represented in all three studies. Overall, this study will be of interest to the Cystic Fibrosis research community because it goes a step further and examines the effect of VX-809 on the interactomes of WT CFTR and four mutants. The study also provides evidence for a phenomenon that has been widely accepted in the field—that VX-809 corrects some CFTR mutants by improving folding, thereby decreasing degradation and resulting in maturation.

**Our response:** We thank reviewer 2 for their constructive feedback. We address all concerns below.

**Reviewer 2, major comment 1:** A general non-trivial concern is that—based on transcriptional profiles and the effects of different proteasome inhibitors on protein correction—different proteasome inhibitors can elicit large changes in mRNAs. Thus, there is concern that some of the effects when proteasome inhibitors are used does not arise solely from inhibition, per se, but from secondary effects.

**Our response:** We agree that this is an important point, because it is well established that proteasome inhibitors can lead to transcriptional remodeling of proteostasis networks. To address the reviewer concerns, we repeated the co-treatment with correctors and proteasome inhibitors for shorter time points, where the translational remodeling of the proteostasis network has not yet occurred. Specifically, we treated with inhibitors and correctors for 6 hours (instead of 16 hours), a time that led to sufficient buildup of P67L band C CFTR to probe the correction. The shorter treatment will eliminate some of the secondary effects because remodeling of the proteostasis environment typically takes longer. For example, some UPR gene transcription occurs at 6 hours but translational upregulation of proteostasis factors takes longer. Importantly, we found that the P67L VX-809 response is still attenuated when treated with bortezomib for only 6 hours. This new data is now shown in Supplemental Figures S9E-H. We updated the text as follows:

"Prolonged bortezomib treatment can lead to considerable remodeling of the proteostasis network, in part through activation of the unfolded protein response. To ensure that the divergent reduction in VX-809 response is directly attributed to proteasomal inhibition and to rule out the possibility of indirect effects, we repeated the dual treatment with VX-809 and bortezomib for shorter duration (6 hours) – sufficient time to allow for CFTR build up but not extensive translational upregulation of proteostasis factors. We find that this shorter treatment also abrogates the VX-809 rescue for P67L (Supplemental Figure 9E-H). These data further suggest a previously unknown role for proteasomal degradation in VX-809 corrections.

**Reviewer 2, major comment 2:** The choice of HEK cells for these studies is questionable, as the general outcome of the mutants and the drugs may not be applicable to other systems. This was not addressed. More generally, HEK cells have robust quality control networks and very
high levels of some chaperones. Thus, the relevance to the mechanism of action of correction in more pertinent systems may not be valid.

**Our response:** Please also see the response to Reviewer 1, Comment 17 and Reviewer 3, Major Comment 1. We used HEK293T cells because stable CFBE cell lines expressing homozygous rare variants, such as P67L and L206W, were not available at the time the study began. We added additional language throughout to make it clear that differences between our results and previous findings may stem from use of different cell lines as well:

“Some difference in identified interactors may be attributed to the use of different cells lines between our study and previous work.”

While the robustness of cellular quality control likely effects the results when compared with CFBE cells, we used HEK293T cells throughout the study and identify only relative changes between CFTR variants in this environment. We have changed language to reflect this limitation as well:

“We also note that use of different cell lines in this study may lead to different identified protein-protein interactions and may explain the observed low degree of overlap between ours and previous data sets.”

Nonetheless, HEK293T are a widely used model system to study protein secretion and many of the protein quality control factors and mechanisms are conserved.

**Reviewer 2, major comment 3:** The authors suggest that their data with E-64 treatment (Figure 6 G and I) confirms that auto-lysosomal degradation is not a major pathway for clearance of P67L and L206W. Yet, they observe an increased abundance of interaction with autophagic factors which is similar (at least for P67L) to the increase observed for F508del (Figure 4B and 5C) in the DMSO-treated samples. This increase is blunted for P67L but not F508del upon correction by VX-809. The authors should clarify that lysosomal degradation is not a major pathway for corrected hyper-responders, and define what they mean by "major pathway."

**Our response:** We thank Reviewer 2 for pointing out this clarification. We deleted the word “major” when comparing degradation pathways because we agree that it is confusing here. We now added clarification that we are referring to corrected CFTR by modifying the sentence as follows:

“E-64 treatment did not result in any accumulation of F508del or P67L band B or band C (Figure 6E-F, Supplemental Figure S9C-D), confirming that auto-lysosomal degradation is not critical for the clearance of these variants under corrector treatment.”

**Reviewer 2, major comment 4:** The authors repeatedly say that VX-809 acts at an inflection point between early stages of biogenesis and routing to degradation pathways. This implies that VX-809 interaction is temporal. This reviewer would suggest that instead, **VX-809 produces an inflection point,** whereby more correctable mutants are folded and therefore shifted toward a more WT-like proteostatic interaction profile. More generally, it is clear that VX-809 acts cotranslationally, based on results from pulse-chase analyses. Thus, this result is not unexpected.
**Our response:** We thank Reviewer 2 for this important suggestion as it makes both the mechanism and writing clearer. We changed “acts at an inflection point” to “VX-809 produces/creates an inflection point” throughout the document. While VX-809 acting co-translationally is well established for F508del, it remained poorly understood for mutants P67L and L206W until this study. We offer the unexpected results that VX-809 fails to completely remodel F508del interactions and present a detailed categorization of pathways and the proteins that VX-809 does remodel for F508del. Indeed, Reviewer 2 pointed out that our study “goes a step further” beyond previous work and “examines the effect of VX-809 on the interactomes of WT CFTR and four mutants.”

**Reviewer 2, major comment 5:** In supplemental figure 4B, more total statistically significant proteostatic interactions with VX-809-treated P67L are observed (69+21+6+8=104), compared to DMSO-treated P67L (54+5+6+21=86). Given the reduction in the overall abundance of interactions (heatmap in figure 4B) especially with later biogenic pathway members, the authors should comment on why they believe that more total proteostatic factors are interacting with the corrected P67L. Does this increase in total numbers of significant interactors upon VX-809 treatment occur for other CFTR variants, or for the WT protein? There are several instances where it appears to this reviewer that either the figures were updated, but the text describing it was not, or perhaps more clarity in what numbers are being compared/contrasted is needed.

**Our response:** Please see our response to Reviewer 1 comment 2, which raised a similar concern. An increased number of interactors does not necessarily imply that that all of those proteins involved are proteostatic interactors. However, we agree with Reviewer 2 that it is worth pointing out that the total number of statistically significant interactors identified for P67L + VX-809 was slightly larger than P67L + DMSO, although we are hesitant to over-interpret these numbers, as they rely on hard p-value cutoffs. It is important to emphasize that for this study the fold changes and protein quantitation are more important than the total number of interactors identified. TMT reporter ion signal allows us to quantify the relative protein abundances for all interactors, even if individual proteins did not pass cutoff filter under one particular conditions. We do not interpret results in terms of number of statistically significant interactors identified, but instead interpret the relative quantitative changes from heatmaps and correlation plots among those interactors. This important point is clarified in the discussion:

“We used AP-MS coupled to TMT labeling, which allows sample multiplexing and direct quantitative comparison of protein interaction changes between mutants and treatments simultaneously.”

**Reviewer 2, major comment 6:** In Figure 2B: The author’s dataset shares 83 interactions (48+35) with the Pankow study, not 93. Also, the Venn diagram shows 388+38+35+48 ID’d proteins for this study, which = 509, not 501 as the text states. And, the Pankow and Hutt datasets share 198 (163+35) interactions out of a total of 1052 total interactions reported for those datasets (392+48+35+163+38+376), which is 18.8% shared, not 30% as stated.

**Our response:** We apologize for the error on these numbers. Please also see Reviewer 1 comment 4 for correction of the 83/93 typo. We also corrected the number of total number of interactors to 509, not 501. For the percent overlap we stated 30% in reference to the overlap of 198 to approximately 600 interactors identified individually in each study (Pankow and Hutt) e.g. in Pankow 198/638 = 31% and in Hutt 198/612 = 32 %. However, Reviewer 2’s comment
pointed out this was unclear and thus we changed the overlap in the text to 18.8% since this is in terms of all the interactors identified in both studies.

**Reviewer 2, major comment 7:** For Supplemental dataset 1, 10 total datasets/runs are represented in the excel file, numbered 1-6 and 10-13, but the 10 datasets/runs are numbered 1-10 in supplemental figures 1 and 2. Are the last 4 datasets the same, and just the numbering changed, or are these actually different datasets?

**Our response:** We thank reviewer 2 for pointing out this error. The last four datasets are indeed the same, but the numbering has changed (e.g. 10 is 7, 11 is 8, 12 is 9, and 13 is 10). We renumbered the data sets in **Supplemental Dataset 1** to reflect the finalized data set numbering system in the final manuscript.

**Reviewer 2, major comment 8:** In Supplemental figure 5E, the text indicates 27 overlapping interactions between the datasets for G85E, however 32 are shown on the figure. Also, the wording is confusing; 32 interactions out of a total of 839 (564+32+243) is only 3.8%, not 13% as stated. If the authors mean to state the percentage of the interactors they identified which overlap, then 32 overlapping out of 275 (32+243) the total = 11.6%. Also, the figure key should read "this study" as in other figures, not Sabusap, to reflect the co-authorship.

**Our response:** See Reviewer 1 comment 7 for correction of 27 overlapping interactors to 32. We were referring to the percentage of overlapping interactors over the total interactors that we identified, which as Reviewer 2 pointed out is 11.6% not 13%. Thus, we changed the reference to **Supplemental Figure S6E** to the following in the text (please note that this corresponds to Supplemental Figure S5 in the former manuscript):

> “From our dataset, 32 G85E interactors overlapped with the previous G85E datasets, albeit the general overlap was relatively small (11.6 % interactors among protein we identified) (**Supplemental Figure S6E**).”

We also updated the key in Supplemental **Figure S6E** to say, “this study”.

**Reviewer 2, minor comments**

**Reviewer 2, minor comment 1:** Only P67L, F508del and WT CFTR are mentioned in the legend for Figure 1, but L206W and G85E are also shown.

**Our response:** Thank you. We added L206W and G85E to the **Figure 1** legend.

**Reviewer 2, minor comment 2:** The authors claim (Fig 1 legend) that the mass spec quantitation (Figure 1D) recapitulates the western blot results (Fig. 1B) but no quantitation is shown for 1B, and for WT treated with VX-809, the amount of band C in the input (1B top panel, lane 4) is reduced vs the DMSO control, but the opposite is true in the IP (1D lane 4).

**Our response:** We added a quantification for **Figure 1B**, specifically the CFTR levels in the IP which are prepared for mass spectrometry quantification in **Figure 1D**. We note there is some
variability amongst VX-809 response between WT in Figure 1B and 1D and the mass spectrometry replicate runs in general. We added the following comment to the Figure 1D legend and referred to Supplemental Figure S2, which shows the individual TMT reporter ion signals for CFTR:

“… Relative mass spec quantification of CFTR levels recapitulates biochemical detection via Western blot (see Supplemental Figure S2 for variability in CFTR levels between replicates).”

Reviewer 2, minor comment 3: Figure 1D: The Y axis title should indicate what is being normalized to WT with DMSO (CFTR mutants).

Our response: We changed the Figure 1D Y axis to: CFTR Quantification (normalized to WT DMSO) to specify what is being normalized.

Reviewer 2, minor comment 4: Figure 6 A and G: the signal to noise for the F508del western blot is poor.

Our response: We replaced Figure 6A and 6G with a different representative blot from our quantification that have a better signal to noise ratio. Please note that panel 6G has been moved to Supplemental Figure S9I.

Reviewer 2, minor comment 5: The text in lines 489-490 is inverted, or incomplete.

Our response: We apologize that the text was confusing or incomplete. We changed this passage as follows to clarify that we can use our method to detect measurable differences in interactors:

“These results highlight that our TMT quantification approach can detect the drug-mediated, reduced interaction between F508del and well-validated protein quality control factors.”

Reviewer 2, minor comment 6: The text in line 498 should be softened to indicate that the P67L interactions shifted toward WT levels instead of “resembled WT levels.”

Our response: Please also see Reviewer 1, comment 2, who also asked us to soften this language. We agree and consequently replaced the word “resembled” with “shifted towards” to soften this statement.

Reviewer 2, minor comment 7: Line 556, change "remodels" to results in.

Our response: We replaced “remodels” with “results in”.

Reviewer 2, cross review: Based on the reviews, I agree with the other reviewers that there is a concern about the choice of the cell line (which we also commented on) as well as the “chicken and egg” problem whether folding/prevention of ERAD allows transport or vice-versa. My other issue is that proteasome inhibitors elicit potent transcriptional responses that alter
proteostatic pathways. It also wasn't clear—as hinted at by reviewer #3—whether this dataset is truly superior to the other published CFTR (corrected or not) mass spec datasets published by Balch and Yates. Finally, I think all reviewers agree that the text is something of a mess—typos, issues with numbers stated in the datasets, etc. If this is to be publishable, substantial work is needed.

Our response: We thank Reviewer 2 for summarizing the overlapping concerns of all three Reviewers. We addressed these concerns about cell lines (Reviewer 1 comment 17, Reviewer 2 comment 2, Reviewer 3 comment 1). We also address the “chicken and egg” problem (Reviewer 2 comment 4, Reviewer 3 comment 5). We do not claim in the text that our dataset is “superior” to any previous published dataset. We clearly highlighted that our dataset contains the interactomes of two new CFTR variants, P67L and L20W, which are of specific biological interest due to their responsiveness to CF therapies. Indeed, all three reviewers commented on the utility of these data. Reviewer 1 was excited about the mechanistic insights offered, Reviewer 2 was enthusiastic about building on the previous studies, and Reviewer 3 highlighted the robustness of quantitative interactomics for making comparisons of interest between mutants.

Reviewer 3

Reviewer 3, general comments: In the current manuscript, McDonald provide a detailed report on the interactome of CFTR bearing four cystic fibrosis-causing mutations, known to induce (either severe or partial) misfolding. The study is performed in HEK293T cells and includes the development and optimization of affinity purification followed by mass spectrometry identification of multiplex samples obtained by combining samples differentially labelled with isobaric tandem mass tags. The study is overall well-designed, and results seem robust - however, the conclusions drawn from data are not fully supported and more caution should be taken when describing what is observed.

Reviewer 3 (Significance): This work advances knowledge on the interactome of CFTR bearing 4 class II mutations. Substantial work has been done in this context, most of which in more relevant cell models (bronchial epithelial cells).

The current work is using a probably more robust method of comparing amount of interactions (tagged approach compared to the non-tagged methodologies previously reported). The work may interest an audience composed not only by CF researchers but all those that focus on protein interactions, protein degradation and trafficking.

The reviewer has large expertise in CFTR biochemistry - trafficking, impact of mutations and assessment of protein-protein interactions.

Our response: Thank you to reviewer 3 for the helpful feedback and positive assessment of our manuscript. We have addressed all comments in detail below.

Reviewer 3, major comment 1: The authors use HEK293T cells, which is not the most relevant cell model to study CF pathophysiology. Most of recent studies (cited by the authors) tend to use the much more relevant CFBE cell line (a bronchial epithelial cell line), as it is much closer
to the "affected organ" in CF. The authors never discuss that aspect, which is, undoubtedly, affecting the overall interactome of CFTR. This is particularly relevant as it has been shown in the past that corrector efficacy is greatly dependent on the cell model used.

**Our response:** Please also see response to Reviewer 1, comment 17 and Reviewer 2, major comment 2. We used HEK293T cells because stable CFBE cells lines expressing homozygous rare variants such as P67L and L206W were not available at the time the study began. We have added additional language throughout to make it clear that differences between our results and previous findings may stem from use of different cell lines as well. For example:

> “Some difference in identified interactors may be attributed to the use of different cells lines between our study and previous work.”

We also added this caveat to the Discussion section:

> “We also note that use of different cell lines in this study may lead to different identified protein-protein interactions and may explain the observed low degree of overlap between ours and previous data sets.”

**Reviewer 3, major comment 2:** A second major aspect has to do with the mutations tested. The authors should discuss their results considering that disease severity differs between the mutations analysed (e.g. whereas 88% of F508del patients are pancreatic insufficient, the number is similar for G85E - 78% - but much lower for P67L and L206W - 33% and 20%, respectively). This of course is related to the levels of rescued protein that can be obtained.

**Our response:** We thank Reviewer 3 for bringing up this data which we did not highlight in the text but is included in Figure 1A.

We added the following sentence to the introduction to highlight this data:

> “All four mutants also demonstrate unique pathobiology in patients which demonstrates responsive mutants P67L and L206W have higher residual function than F508del and G85E (Figure 1A).”

Furthermore, we also added the following sentence to the Discussion to evaluate our results in the context of this alternative interpretation e.g. that protein levels may contribute to rescue of hyper-responsive variants just like conformational defect may contribute to rescue:

> “Alternatively, protein levels may contribute to rescue of hyper-responsive variants. P67L and L206W demonstrate higher residual function in patients (Figure 1A) and thus may also present higher levels of protein available for rescue at steady state.”

**Reviewer 3, major comment 3:** A similar comment applies to the inclusion of both F508del and G85E is a so-called hypo-responsive group - however, G85E is a non-responder and F508del is a responder (although a low responder). This is quite a difference by itself.

**Our response:** We agree with Reviewer 3 that these two mutants show distinct responsiveness to VX-809. Consequently, we have revised our description of F508del as moderate-responsive and G85E as non-responsive throughout the text. For instance:
An interesting distinction between the moderate and non-responsive variants is that F508del displays some reductions in proteostasis interactions with folding and translation components when treated with VX-809 (Figure 4C), while G85E interactions are completely refractory to the corrector compound (Figure 5D).

**Reviewer 3, major comment 4**: Results suggest relevant differences in several proteostasis components, among mutants, and above all after correction. This relies only on MS data, although in such a study, there should be some sort of validation (e.g. by WB) for a few selected "interactors" that are illustrative of the mechanisms that the authors decide to highlight.

**Our response**: We agree with Reviewer 3 and have confirmed select interaction changes by Western blots on IP samples for F508del and P67L under DMSO and VX-809 conditions. One of these interactors, PSMC3, was identified as important in this study specifically. This new data was added as Supplemental Figure S5 and highlighted as follows:

“To further support our model that VX-809 creates an inflection point for P67L but not for F508del, we validated interaction levels for key folding and degradation proteins co-IP followed by Western blot. Well characterized CFTR interactors BAG2, Derlin-1, and CANX were chosen for their known role in folding and degradation\(^{44,46,48}\). We also monitored PSMC3, whose interactions with P67L was more strongly reduced by VX-809 than with F508del (Figure 4E). Consistent with our interactomics data, VX-809 significantly reduced P67L interaction with BAG2, Derlin-1, CANX, and PSMC3 (Supplemental Figure 5). By contrast, VX-809 failed to reduce F508del interaction with BAG2, Derlin-1, CANX, or PSMC3 (Supplemental Figure 5).

**Reviewer 3, major comment 5**: There is a general claim (very clear e.g in lines 401-402) that treatment with VX-809 leads to changes in the interactions and this causes correction. It is however more likely that the opposite is occurring - the compound is specifically correcting CFTR misfolding allowing the protein to escape ER retention and thus reshaping its interactome. This needs to be properly discussed and adjusted.

**Our response**: Please also see our response to Reviewer 2, major comment 4. Here Reviewer 2 suggested changing the language from “VX-809 acts at an inflection point” to “VX-809 produces an inflection point.” We took Reviewer 2’s advice and made these changes throughout the text including in the specific passage brought up by the reviewer. Reviewer 2 also referred to this point as the “chicken and egg problem” in the cross-review. We agree that we cannot distinguish VX-809 binding directly influencing proteostasis interaction changes, or whether these interaction changes are reflective of the enhanced folding and stability of the drug bound CFTR molecule. This point is clarified in the discussion:

“Thus, VX-809 hyper-responsive CFTR mutants most likely reach a correctable conformation before the checkpoint to route for proteosomal degradation.”

During the revision of this manuscript, several papers were published converging on a common binding site for VX-809, thus definitively demonstrating that VX-809 does directly bind to CFTR. Thus, in addition to changing the language above, we included the following sentence in the introduction to highlight these recent discoveries with the appropriate citations:
Furthermore, two studies recently converged on a putative VX-809 binding site to CFTR TMD126,27.

**Reviewer 3, major comment 6:** Considering the correlation observed in Fig. 3C, the observed increases shown in Fig. 3D were expected. How does this compare with interactions from other pathways (e.g. ones no so obviously connected to CF)?

**Our response:** We thank Reviewer 3 for pointing this out, and we agree that upregulation is expected given the overall correlation observed in **Figure 3C**. However, the magnitude of upregulation depends on whether the pathway in question is involved in CF biogenesis. To demonstrate this, we plotted the interactor quantitation distributions for folding vs. pathways for pathways less obviously connected to CF: cytoskeleton, differentiation, metabolism, and nuclear import/export. We plotted these comparisons for both F508del and P67L and added them to **Supplemental Figure S4** with a detailed description of the plots and interpretation included in the Supplemental Figure captions. We found folding components increase compared to WT with a generally sharper and slightly higher magnitude distribution than the upregulation of unconnected pathways. We also added the following to the main text to draw attention to this important difference:

> “Notably, pathways important for CFTR biogenesis, such as folding, show a shaper distribution of log 2 fold change compared to pathways less clearly associated with CFTR biogenesis (**Supplemental Figure S4B-C**).”

**Reviewer 3, major comment 7:** The grouping by category (e.g. Fig. 4C) was apparently made "manually". How to be sure that this procedure isn't introducing a bias by combining what seems "more appropriate"?

**Our response:** We performed an initial classification of proteins into pathways based on the categories established in the prior CFTR interactomics study by Pankow, et al. We then classified newly identified proteins by relevant GO terms related to those initial classifications. We clarified the methods section to emphasize that initial categorization was based on previous studies:

> “First, we searched our dataset against the pathway classification presented by Pankow et al30. This step classified all previously identified interactors. For novel interactors we manually annotated pathways by searching gene names on UniProt and classifying pathways by GO-terms for Biological Pathway.”

**Reviewer 3, major comment 8:** Can the authors confirm the identity of lower molecular weight band that they call "band A"? Due to the cotranslational mechanism of glycosylation, it is very unlikely that unglycosylated CFTR is detected. This band, observed under certain conditions that promote accumulation, is most likely a band previously described as resulting from alternative translation initiation, which is more abundant in mutants such as F508del.

**Our response:** We thank Reviewer 3 for pointing this out. We have removed the distinction of Band B and Band A from the paper entirely, as we do not find this distinction important for the analysis or interpretation of our results. We simply changed references to these bands to “immature CFTR”, where necessary. We also changed the **Figure 6** and **Supplemental Figure S8** axes labels from “A/B band” to “immature CFTR” to reflect this change.
Reviewer 3, minor comments

Reviewer 3, minor comment 1: Please bear in mind that the concept "theratype" relates to the type of approach needed for correction and not to any specific compound/drug. In the case of the mutations studied here, they belong to same theratype - as what is needed is to increase CFTR quantity.

Our response: We politely disagree with Reviewer 3. See Clancy, J. P. et al. CFTR modulator theratyping: Current status, gaps and future directions. J. Cyst. Fibros. 18, 22–34 (2019). Under the section “Theratype definition” see “More recently the term has been used to describe an approach to characterize mutations by their response to CFTR modulators across various model systems, which can include functional and biochemical characterization.” We clarify our definition of theratype in the introduction where we cite the aforementioned paper:

“Despite the identical classification of F508del, P67L, L206W, and G85E variants as class-II (abnormal protein folding/trafficking)22, this classification scheme provides little basis for predicting CFTR therapeutic response, otherwise known as theratype23,24.”

Reviewer 3, minor comment 2: From the heat maps presented, it seems that all the interactions are increased by mutation. There is very unlikely - in previous studies, it was shown that some interactions increase while other decrease. This needs to be discussed.

Our response: We call attention to Supplemental Figure S3B which shows the entire heatmap for all proteins in all pathways considered and demonstrates downregulation in several mutants, especially WT, P67L, and L206W with VX-809. However, we thank Reviewer 3 for bringing attention to this issue and we added the following sentences to the discussion to highlight this point and cite the appropriate work:

“Most of the interactions presented in this study were upregulated compared to WT with DMSO. The exceptions include WT and L206W with VX809 (Supplemental Figure S3A, S3B and Figure 5C). This is in contrast to previous studies which showed upregulation and down regulation of interactors compared to WT28,62.”
Dear Dr. Plate:

I've assessed the revised manuscript myself and find the changes adequately address reviewers' concerns. I ask only that you indicate the n for the violin plots so the reader can appreciate the size of the dataset being considered.

Sincerely,
Elizabeth Miller
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Plate,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) 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 include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter"). Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

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Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
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Response to Editor Comments

Monitoring Editor comments:

Dear Dr. Plate:

I've assessed the revised manuscript myself and find the changes adequately address reviewers' concerns. I ask only that you indicate the n for the violin plots so the reader can appreciate the size of the dataset being considered.

Our response: Thank you, Dr. Miller, for taking the time to evaluate our revised manuscript yourself and for your positive assessment. We revised the violin plots in Figure 3D, Figure 4C, and Figure 5D to now highlight the n (number of proteins) included in each of the pathway dataset. The raw data is also included in Supplemental Dataset 4, which we highlighted in the figure legends:

“n represents the number of identified proteins in the considered pathway (Supplemental Dataset 4).”
Dear Dr. Plate:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Sincerely,
Elizabeth Miller
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Plate:

Congratulations on the acceptance of your manuscript.

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