A small-molecule inhibitor and degrader of the RNF5 ubiquitin ligase

Jingjing Ruan, Dongdong Liang, Wenjing Yan, Yongwang Zhong, Daniel Talley, Ganesha Rai, Dingyin Tao, Christopher LeClair, Anton Simeonov, Yinghua Zhang, Feihu Chen, Nancy Quinney, Susan Boyles, Deborah Cholon, Martina Gentzsch, Mark Henderson, Fengtian Xue, and Shengyun Fang

Corresponding author(s): Shengyun Fang, University of Maryland, Baltimore

Review Timeline:

Submission Date:	2022-06-29
Editorial Decision:	2022-08-08
Revision Received:	2022-08-19
Accepted:	2022-08-25

Editor-in-Chief: Matthew Welch

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

RE: Manuscript #E22-06-0233
TITLE: A small-molecule inhibitor and degrader of the RNF5 ubiquitin ligase

Dear Dr. Fang:

Your manuscript has now been reviewed by two experts, both of whom are supportive of the publication and recognise the contribution your work will make to the field. Both reviewers have comments that should be easy to address with textual changes. In some cases, additional data would certainly improve the impact/reach of the findings, and if you have such data in hand, or can complete the experiments in a timely manner, I would encourage you to do this. Reviewer 2 in particular, suggests several experimental approaches that would clarify the study, including better delineating the relationship between RNF5 and HRD1, and providing some clarity on the link between abrogation of Ubiquitination and degradation (which might well be linked to the aforementioned point). If experiments are not readily available for these points, I encourage you to clarify the issues in the discussion.

Sincerely,

Elizabeth Miller
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Fang,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments 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 haveopted out of publishing the review history.

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

Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

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 manuscript, and figures, use this link: Link Not Available

Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

RNF5 is an endoplasmic reticulum (ER) membrane localized RING finger E3 ubiquitin ligase that plays a role in targeting non-native proteins for ER-associated degradation (ERAD). These proteins include a number of disease-associated proteins including CFTR, which is associated with cystic fibrosis. Therefore, it is an important drug target for possible therapeutics. This manuscript described the discovery and comprehensive characterization of an inhibitor to RNF5, an enzo**thiophene derivative, FX12. FX12 acts by directing binding to RNF5 (as shown by surface plasmon resonance analysis) and targeting it for degradation in cells. Ample evidence is provided to support the model that FX12 inhibits the dislocation of ERAD substrates by binding to FX12. While FX12 treatment was able to efficiently release the important disease associated CFTR mutant (F508) from the ER, its utility fell short when monitoring the activity of the CFTR- F508 at the surface of patient primary human bronchial epithelial (HBE) cells. A previous inhibitor of RNF5 (but not a degrader of RNF5), inh-2, has been shown to increase CFTR activity by inhibiting FX12. However, the value in this study rests with the thoroughness of the characterization of FX12 actions as an RNF5 inhibitor in vitro and in cells, its unique mechanism of action by acting as a degrader of RNF5 and the potential for additional derivatives for future therapeutics.**

Overall, the manuscript is well written and organized. The experiments are cleverly designed for a thorough and well controlled characterization of FX12 action.

Minor issues to address:
Figure 3A, what is the nature of the doublet? Which band is RNF5?

Can you discuss in more detail which arm(s) of the UPR are activated by FX12 treatment? Which ones are not? and its implications.

All conditions include BTZ in Figure 8C. What about FX12 alone?

Quantitation for Figure 8F should be provided.

Reviewer #2 (Remarks to the Author):

OVERVIEW
In this manuscript, the authors describe a small molecule that is proposed to both inhibit and degrade the ER-resident ubiquitin ligase RNF5. RNF5 has been implicated as an important factor in diseases ranging from cystic fibrosis to various forms of cancer, providing an impetus for the development of tool compounds and therapeutic agents targeting its activities. Derivatives of the existing LOPAC compound Stattic yielded FX12, a compound with reduced selectivity towards STAT3 and overall cytotoxicity but provided data that it still impacted protein dislocation from the ER, as determined by a complementation assay built around split GFP and the retrotranslocation of a misfolded glycoprotein NHK (Figure 1). The authors provide data that FX12 prevents bortezomib (BTZ)-induced accumulation of complemented GFP, and suggest a defect in NHK retrotranslocation, supported by reduced NHK ubiquitination and degradation rate as determined by IP-WB and cycloheximide chase respectively (Figure 2). They show that FX12 causes a dose and time-dependent reduction of endogenous RNF5 protein degraded by a pathway involving VCP/p97 and the proteasome, as well as increased polyubiquitin conjugation (of an exogenously expressed form). RNF5 mRNA levels are unchanged, indicating the effect is post-transcriptional and leading them to conclude that turnover occurs via ERAD. FX12 may induce the UPR, as indicated by elevated levels of Ube2j1 and BiP (Figure 3). In vitro binding assays presented indicate high nanomolar affinity of FX12 for the N-terminal domain of RNF5 as well as a dose-dependent, selective impairment of RNF5 auto-ubiquitination. FX12 does not affect E2 loading of Ub (Fig 4F,G) and is phenocopied by expressing a ubiquitination-defective RNF5 mutant.

Using thermal shift assays, the authors observe increase stability of both RNF5 and Derlin1, suggesting direct engagement, although higher FX12 concentrations were used, (20μM/1hr). RNF5 was also stabilized in Derlin1 KO cells (but bands appear weaker than WT, Fig 5E vs A). The authors turn to CFTR∆F508 to assess effects of FX12 on maturation, excitingly showing that co-application with clinical compounds promoting folding/trafficking increases the amount of B (ER) and C (surface) forms of ∆F508 as well as wild type CFTR (Figure 6). FX12 decreases GFP-∆F508 ubiquitination in a dose-dependent manner while inhibiting its degradation (Fig 6F and G), suggesting that it results in more available to fold and mature. The impact of FX12 on HBE cultures was less clear, with no increase in CFTR activity or surface protein observed with FX12 treatments (Figure 7). To confirm that FX12 acted at RNF5, the authors treated drGFP (NHK) WT and siRNF5 cells with FX12 + Btz and observed that cells lacking RNF5 continued to accumulate GFP, even without RNF5.

Overall, the manuscript presents some intriguing observations, a potentially useful tool compound, and its aims are

highly relevant for the ER field and beyond. The findings are suitable in scope for this journal. While some of the data are compelling, others are confusing and lack the additional detail/follow up experiments that would have provided needed clarity. The manuscript is clearly written and appropriately referenced. This reviewer tries not to request additional experiments that would unnecessarily prolong time to publication, but in this instance, they may be beneficial. In this reviewer's view, there would be several points that require attention in order to solidify the foundations of the manuscript. They are listed below.

SELECTED ISSUES

Issue 1. The authors provide data demonstrating their NHK-drGFP reporter has its dislocation, or more accurately, retrotranslocation, impaired by FX12 and then provide data implicating the loss of RNF5 ubiquitination activity (Fig. 4H) in this. Ubiquitination of NHK is also reduced by FX12, which could suggest RNF5 is ubiquitinating NHK. Yet there is an abundance of data in the literature (including from the authors themselves) demonstrating ERAD of NHK depends on Hrd1, which is mentioned in the discussion. Moreover, data demonstrating siRNF5 has only minor/modest impact on NHK-drGFP accumulation would seem to indicate that RNF5 does not play a role in NHK turnover (Figure 8B,C). While discussed in the text, the speculation that RNF5 activity somehow indirectly impacts soluble glycoprotein degradation via Hrd1, really needs to be demonstrated experimentally in a more direct manner. This reviewer would appreciate greater clarity of the apparent relationship between RNF5 and Hrd1.

Issue 2. In Figure 4E, the authors clearly demonstrate that FX12 at 5μM blocks RNF5-mediated auto-ubiquitination in vitro. Yet in Figure 3A, FX12 is seen to enhance degradation and Btz and CB-5083 effects would indicate that occurs via ERAD. And in Figure 3E, FX12 appears to increase RNF5 polyubiquitination, which is consistent. This would suggest that 1) either ubiquitination is required for RNF5 stability and without it, the protein is destabilized leading to the effects of FX12 or 2) that RNF5 is not auto-ubiquitinated but rather is the substrate of another ER-E3.. perhaps MARCH6, gp78 or Hrd1. In either case, resolving this apparent conundrum of attenuated ubiquitin conjugation activity and increased degradation would be necessary to better define how FX12 exerts it mode of action. In this reviewer's opinion, additional data to help clarify this would substantially add to the impact of the findings. At the very least, perhaps they can comment or provide some rationalization.

Issue 3. Figure 2C would seem to indicate that FX12 is capable of blocking NHK degradation, while correlative data of Fig 2A would indicate that NHK remains in the ER but is not shown directly. How is this known? Could the authors confirm this by demonstrating continued sensitivity of NHK-drGFP to EndoH during FX12 treatment? This study would benefit from better kinetic analyses of NHK in the form of radiolabeled pulse-chase assays but perhaps this classic technique may not be easily undertaken in the authors' institution.

SPECIFIC COMMENTS/QUESTIONS

Comment 1. In Figure 8C, it is not clear why for this assay the treatment times were 23 hrs when for other drGFP assays (e.g. Figure 2A) they are only 6 hr. Could the authors explain why an additional 17 hrs was required to generate a signal or was this just to allow more time for the siRNAs to take effect?

Comment 2. Is the RNF5 amount limiting for degradation? In Figure 4H, would overexpression of FLAG-RNF5 WT be anticipated to accelerate NHK degradation rates? Would this be a more appropriate control for FLAG-RNF5-RINGm?

Comment 3. The lack of effect of FX12 on CFTR△F508 maturation in human bronchial epithelial cultures was disappointing, particularly when compared to the GFP-tagged forms in BHK cells. Are there any notable differences in RNF5 expression between these 2 cell systems that might provide an explanation for this discrepancy?

Comment 4. In Figure 6C, it is not clear why there is a difference in the levels of B and C bands between lanes 1 and 10, or lanes 4 and 11, when these should be the same conditions. Is this just experimental variation or changes in detection sensitivity?

Comment 5. While this reviewer appreciates the denaturation step introduced prior to IPs and ubiquitin western blots (e.g. Fig 2B, 3E, 6F), treatment with a recombinant DUB remains the gold standard confirming ubiquitin conjugation to substrates. If the authors have these data, perhaps they might include it in their figures.

Responses to the reviewers:

: Manuscript #E22-06-0233
TITLE: A small-molecule inhibitor and degrader of the RNF5 ubiquitin ligase

---

Reviewer #1 (Remarks to the Author):

RNF5 is an endoplasmic reticulum (ER) membrane localized RING finger E3 ubiquitin ligase that plays a role in targeting non-native proteins for ER-associated degradation (ERAD). These proteins include a number of disease-associated proteins including CFTR, which is associated with cystic fibrosis. Therefore, it is an important drug target for possible therapeutics. This manuscript described the discovery and comprehensive characterization of an inhibitor to RNF5, an enzothiophene derivative, FX12. FX12 acts by directing binding to RNF5 (as shown by surface plasmon resonance analysis) and targeting it for degradation in cells. Ample evidence is provided to support the model that FX12 inhibits the dislocation of ERAD substrates by binding to FX12. While FX12 treatment was able to efficiently release the important disease associated CFTR mutant (DF508) from the ER, its utility fell short when monitoring the activity of the CFTR-DF508 at the surface of patient primary human bronchial epithelial (HBE) cells. A previous inhibitor of RNF5 (but not a degrader of RNF5), inh-2, has been shown to increase CFTR activity by inhibiting FX12. However, the value in this study rests with the thoroughness of the characterization of FX12 actions as an RNF5 inhibitor in vitro and in cells, its unique mechanism of action by acting as a degrader of RNF5 and the potential for additional derivatives for future therapeutics.

Overall, the manuscript is well written and organized. The experiments are cleverly designed for a thorough and well controlled characterization of FX12 action.

Minor issues to address:
Figure 3A, what is the nature of the doublet? Which band is RNF5?

We thank the reviewer for their assessment of our work and helpful comments.

We appreciate this reviewer calling attention to this carelessness. The up band for the two RNF5 immunoreactive bands in Figure 3A is now indicated with an asterisk because it is non-specific. RNF5, which was downregulated by FX12 as evidenced by a number of subsequent experiments (Figure 3B, D, F and Figure 6A); is represented by the lower band. Data in Figure 3A is an early experiment for this project that used a rabbit polyclonal anti-RNF5 antibody from Invitrogen (#PA5-71703), which recognizes a non-specific band. A mouse monoclonal anti-RNF5 antibody from Santa Cruz Biotech (# sc-81716), which specifically recognizes RNF5, was employed in the studies that followed.

Can you discuss in more detail which arm(s) of the UPR are activated by FX12 treatment? Which ones are not? and its implications.

We have added additional data to Fig 3A examining the effects of FX12 on expression of the UPR sensors PERK, Ire1a and ATF6. We observed subtle changes in mobility indicative of

activated UPR. In conjunction with results for the UPR regulated genes BiP, Ube2j1, and Derlin1 (strong upregulation by compound), the results suggest that all three UPR arms were activated to some extent, but overall to a lesser degree as compared with tunicamycin treated cells. We have also clarified this in the text in the Results section (page 7, lines 7-9).

All conditions include BTZ in Figure 8C. What about FX12 alone?

The drGFP assay requires proteasomal inhibition to generate fluorescence in the cytoplasm (otherwise the dislocated protein is rapidly degraded), therefore it is an essential component to perform the dislocation experiment. Without proteasome inhibitor, the drGFP cells show no GFP signal (with or without FX12).

Quantitation for Figure 8F should be provided.

Quantitation for Figure 8F (now Figure 9C in revision) has been added, please see Figure 9D-E.

Reviewer #2 (Remarks to the Author):

OVERVIEW
In this manuscript, the authors describe a small molecule that is proposed to both inhibit and degrade the ER-resident ubiquitin ligase RNF5. RNF5 has been implicated as an important factor in diseases ranging from cystic fibrosis to various forms of cancer, providing an impetus for the development of tool compounds and therapeutic agents targeting its activities. Derivatives of the existing LOPAC compound Stattic yielded FX12, a compound with reduced selectivity towards STAT3 and overall cytotoxicity but provided data that it still impacted protein dislocation from the ER, as determined by a complementation assay built around split GFP and the retrotranslocation of a misfolded glycoprotein NHK (Figure 1). The authors provide data that FX12 prevents bortezomib (BTZ)-induced accumulation of complemented GFP, and suggest a defect in NHK retrotranslocation, supported by reduced NHK ubiquitination and degradation rate as determined by IP-WB and cycloheximide chase respectively (Figure 2). They show that FX12 causes a dose and time-dependent reduction of endogenous RNF5 protein degraded by a pathway involving VCP/p97 and the proteasome, as well as increased polyubiquitin conjugation (of an exogenously expressed form). RNF5 mRNA levels are unchanged, indicating the effect is post-transcriptional and leading them to conclude that turnover occurs via ERAD. FX12 may induce the UPR, as indicated by elevated levels of Ube2j1 and BiP (Figure 3). In vitro binding assays presented indicate high nanomolar affinity of FX12 for the N-terminal domain of RNF5 as well as a dose-dependent, selective impairment of RNF5 auto-ubiquitination. FX12 does not affect E2 loading of Ub (Fig 4F,G) and is phenocopied by expressing a ubiquitination-defective RNF5 mutant.

Using thermal shift assays, the authors observe increase stability of both RNF5 and Derlin1, suggesting direct engagement, although higher FX12 concentrations were used, (20μM/1hr). RNF5 was also stabilized in Derlin1 KO cells (but bands appear weaker than WT, Fig 5E vs A). The authors turn to CFTRΔF508 to assess effects of FX12 on maturation, excitingly showing that co-application with clinical compounds promoting folding/trafficking increases the amount of B (ER) and C (surface) forms of ΔF508 as well as wild type CFTR (Figure 6). FX12 decreases GFP-ΔF508 ubiquitination in a dose-dependent manner while inhibiting its degradation (Fig 6F

and G), suggesting that it results in more available to fold and mature. The impact of FX12 on HBE cultures was less clear, with no increase in CFTR activity or surface protein observed with FX12 treatments (Figure 7). To confirm that FX12 acted at RNF5, the authors treated drGFP (NHK) WT and siRNF5 cells with FX12 + Btz and observed that cells lacking RNF5 continued to accumulate GFP, even without RNF5.

Overall, the manuscript presents some intriguing observations, a potentially useful tool compound, and its aims are highly relevant for the ER field and beyond. The findings are suitable in scope for this journal. While some of the data are compelling, others are confusing and lack the additional detail/follow up experiments that would have provided needed clarity. The manuscript is clearly written and appropriately referenced. This reviewer tries not to request additional experiments that would unnecessarily prolong time to publication, but in this instance, they may be beneficial. In this reviewer's view, there would be several points that require attention in order to solidify the foundations of the manuscript. They are listed below.

SELECTED ISSUES

Issue 1. The authors provide data demonstrating their NHK-drGFP reporter has its dislocation, or more accurately, retrotranslocation, impaired by FX12 and then provide data implicating the loss of RNF5 ubiquitination activity (Fig. 4H) in this. Ubiquitination of NHK is also reduced by FX12, which could suggest RNF5 is ubiquitinating NHK. Yet there is an abundance of data in the literature (including from the authors themselves) demonstrating ERAD of NHK depends on Hrd1, which is mentioned in the discussion. Moreover, data demonstrating siRNF5 has only minor/modest impact on NHK-drGFP accumulation would seem to indicate that RNF5 does not play a role in NHK turnover (Figure 8B,C). While discussed in the text, the speculation that RNF5 activity somehow indirectly impacts soluble glycoprotein degradation via Hrd1, really needs to be demonstrated experimentally in a more direct manner. This reviewer would appreciate greater clarity of the apparent relationship between RNF5 and Hrd1.

We also thank reviewer 2 for their positive assessments and for pointing out areas that were confusing where clarity could be improved in the revision.

In agreement with our observations through the discovery of FX12, another recent study reported that RNF5 is an E3 for Hrd1, thereby inhibiting Hrd1-mediated ERAD (Yang, Q. et al. Hepatology. 2021:3018-3036). Consistently, we did see an increase in NHK-drGFP in siRNF5 cells (Figure 8B, statistical analysis has been added). FX12 affects RNF5 function by 1) inhibiting its E3 activity (Figure 4E-G) and changing its interactions with Hrd1 complex proteins such as Derlin1 and Derlin2 (Figure 5H), and maybe others. This reviewer indeed raised an important and complex question regarding the precise relationship between RNF5 and Hrd1, which will be investigated in our future studies.

Issue 2. In Figure 4E, the authors clearly demonstrate that FX12 at 5μM blocks RNF5-mediated auto-ubiquitination in vitro. Yet in Figure 3A, FX12 is seen to enhance degradation and Btz and CB-5083 effects would indicate that occurs via ERAD. And in Figure 3E, FX12 appears to increase RNF5 polyubiquitination, which is consistent. This would suggest that 1) either ubiquitination is required for RNF5 stability and without it, the protein is destabilized leading to the effects of FX12 or 2) that RNF5 is not auto-ubiquitinated but rather is the substrate of another ER-E3.. perhaps MARCH6, gp78 or Hrd1. In either case, resolving this apparent conundrum of attenuated ubiquitin conjugation activity and increased degradation would be necessary to better define how FX12 exerts it mode of action. In this reviewer's opinion,

additional data to help clarify this would substantially add to the impact of the findings. At the very least, perhaps they can comment or provide some rationalization.

We agree with the reviewer that ubiquitination by an ER ubiquitin ligase is involved in FX12-induced RNF5 degradation and identification of the E3 would more fully define the mechanism of action for FX12. Identification of the E3 responsible for RNF5 degradation is not a trivial task experimentally, as there are many E3 ubiquitin ligases, in addition to the three E3s rightly indicated as candidates by the reviewer, which regulate ERAD. Identification of the E3 and understanding how this/these E3(s) mediate RNF5 degradation in response to FX12 will be better resolved, in our opinion, in a separate study.

Issue 3. Figure 2C would seem to indicate that FX12 is capable of blocking NHK degradation, while correlative data of Fig 2A would indicate that NHK remains in the ER but is not shown directly. How is this known? Could the authors confirm this by demonstrating continued sensitivity of NHK-drGFP to EndoH during FX12 treatment? This study would benefit from better kinetic analyses of NHK in the form of radiolabeled pulse-chase assays but perhaps this classic technique may not be easily undertaken in the authors' institution.

Multiple lines of evidence reported in our previous study indicate that NHK-drGFP fluorescence faithfully reflects NHK dislocation or retrotranslocation (Zhong Y and Fang S, JBC, J Biol Chem 2012: 28057-66). FX12 inhibits NHK ubiquitination and degradation (Figure 2B, C), which is in line with an inability of NHK to undergo dislocation. In addition, we did not see molecular weight drop of NHK, a reflection of deglycosylation of dislocated NHK, in FX12-treated cells (Figure 2C). The previous study and current data support that the stabilized NHK remains in the ER.

SPECIFIC COMMENTS/QUESTIONS

Comment 1. In Figure 8C, it is not clear why for this assay the treatment times were 23 hrs when for other drGFP assays (e.g. Figure 2A) they are only 6 hr. Could the authors explain why an additional 17 hrs was required to generate a signal or was this just to allow more time for the siRNAs to take effect?

Yes, we used long treatment time to ensure that RNF5 was completely silenced during FX12 treatment. This was done since our preliminary studies found that FX12 still inhibited drGFP, while to a lesser degree, when residual RNF5 presented.

Comment 2. Is the RNF5 amount limiting for degradation? In Figure 4H, would overexpression of FLAG-RNF5 WT be anticipated to accelerate NHK degradation rates? Would this be a more appropriate control for FLAG-RNF5-RINGm?

We used RNF5-RINGm was to mimic FX12-mediated inactivation of RNF5 E3 activity (Figure 4E-G) to determine whether the E3-inactive RNF5 inhibits NHK degradation. RNF5 negatively regulates Hrd1-mediated ERAD (Yang, Q. et al. Hepatology. 2021:3018-3036) (Figure 8B). Overexpression of RNF5 WT is expected to inhibit NHK degradation.

Comment 3. The lack of effect of FX12 on CFTRΔF508 maturation in human bronchial epithelial cultures was disappointing, particularly when compared to the GFP-tagged forms in BHK cells. Are there any notable differences in RNF5 expression between these 2 cell systems that might provide an explanation for this discrepancy?

We too were surprised to not see a strong functional effect in the HBE cells. Yes, it is possible that RNF5 may differ between these cell types and possibly have an adverse effect on other components of ERAD complexes. At this point, however, this is speculative. This discrepancy warrants further investigation, which we have also attempted to convey in the text (see Discussion section, page 15, lines 13-18).

Comment 4. In Figure 6C, it is not clear why there is a difference in the levels of B and C bands between lanes 1 and 10, or lanes 4 and 11, when these should be the same conditions. Is this just experimental variation or changes in detection sensitivity?

These are different experiments, with exposure times on the two blots selected to capture signal across the samples. While band intensity is not identical, the effect of 5 uM FX12 are the same for lanes 1vs4 and 10vs11, with an increase in band B and appearance of small amount of band C. The experiments were to determine whether FX12 could enhance the effects of both VX809 and VX661 (which is demonstrated by the results); however, it is not possible to directly compare VX809 and VX661 in these two independent experiments.

Comment 5. While this reviewer appreciates the denaturation step introduced prior to IPs and ubiquitin western blots (e.g. Fig 2B, 3E, 6F), treatment with a recombinant DUB remains the gold standard confirming ubiquitin conjugation to substrates. If the authors have these data, perhaps they might include it in their figures.

We do not have data using DUB-treated lysates.

RE: Manuscript #E22-06-0233R
TITLE: "A small-molecule inhibitor and degrader of the RNF5 ubiquitin ligase"

Dear Dr. Fang:

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. Fang:

Congratulations on the acceptance of your manuscript.

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

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

Would you like to see an image related to your accepted manuscript on the cover of MBoC? Please contact the MBoC Editorial Office at mboc@ascb.org to learn how to submit an image.

Authors of Articles and Brief Communications are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

We are pleased that you chose to publish your work in MBoC.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org
