SARS-CoV-2 ORF10 impairs cilia by enhancing CUL2 ZYG11B activity

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September 23, 2021

Re: JCB manuscript #202108015

Prof. Wei Li
Chinese Academy of Sciences

Dear Prof. Li,

Thank you for submitting your manuscript entitled "SARS-CoV-2 ORF10 impairs cilia by enhancing CUL2 ZYG11B activity". Your manuscript has been assessed by expert reviewers, whose comments are appended below. We thank you for your patience while we were assessing the reviews. Although the reviewers are overall enthusiastic about this work, they have raised several significant concerns that unfortunately preclude publication of the current version of the manuscript in JCB. We hope that you will be able to address these concerns in a revised manuscript.

In particular, a successful revision would require that you compare your mass spectrometry results with those from the Elledge lab and provide some compelling discussion on why they differ, as requested by all three reviewers (rev #1 p1; rev #2 general comments & rev #3 p6) and that you demonstrate that ORF10 enhances proteasomal degradation via the CUL2(ZYG11B) complex using the assay from Mena et al., as suggested by reviewer #2. In addition, more mechanistic insights into how the ORF10-CUL2(ZYG11B) interaction affects IFT46 protein degradation are needed (rev #1 p6 & rev #2 p3), as well as addressing all technical issues concerning the in vivo experiments (rev #3 main comment, p1-2). Finally, you are encouraged to pursue the validation of your main findings in a motile, multiciliated cell culture model, as noted by reviewers #2 and #3 (rev #2 general comments & rev #3 p11). We also hope that you will be able to address each of the reviewers' other issues as well.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

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If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised. You can contact the journal office with any questions, cellbio@rockefeller.edu.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Maxence Nachury
Monitoring Editor
Journal of Cell Biology

Lucia Morgado-Palacin, PhD
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In this manuscript the authors study the previously identified interaction between SARS-COV-2 ORF10 and the ubiquitin E3 ligase adapter ZYG11B. They confirm the interaction by pull-down assays and find that it extends to the E2 enzyme UBE2D1. They then test for protein changes upon ORF10 expression using TMT mass spectrometry and identify a series of proteins involved in cilia biogenesis and maintenance that have reduced abundance after ORF10 expression. After confirming this for a small subset they focus on one protein, IFT146 where they show more detailed effects of ubiquitination.
They then study cilia formation in the presence of ORF10 on 5 cells and see that ZYB11b is important for this. Finally they analyze the cilia in mice that are infected with a spike and ORF10 containing lentivirus, where again they observe an effect on cilia.

A new example of an E3-ligase adapter being hijacked to a set of new targets is of general interest scientifically and if this underlies the loss of smell and taste observed in COVID-19 it would have a very broad impact. However, exactly because of its potential implications it is important to solidify the data. At this point there are a number of concerns and aspects that require better controls and more solid data.

a) A major concern is that an earlier paper by Steve Elledge and with involvement from the Gygi lab did a similar TMT proteomics experiment, also on 293T cells, but did not observe the differences in cilia-related proteins mentioned here. The authors do mention this, but seem to treat it rather lightly, whereas I find it a real concern, as the Gygi lab has very well-controlled proteomics, that I would trust over most others in the world. It would be good to provide a more detailed comparison with the outcome of the Gygi/Elledge experiment to see if there is an explanation for this difference. One could imagine that the levels of ORF10 are important, but then it would be good to analyze this carefully

b) The difference extends to the fact that the Gygi/Elledge analysis also did not observe a difference in the normal function of ZYG11B, which would also contradict a wholesale redirection of the targeting of ZYG11B. If the authors suggest here a new series of targets for ZYG11B, it would be good to show how ZYG11B affects its usual targets under these conditions. Or do the ciliated proteins have the ZYG11B degrons?

c) The authors propose a three-way interaction between ORF10, ZYG11B and UBE2D1, but only show individual pull-downs. These are small peptides or easily produced proteins (see e.g. the recent structural data on ZYG11b), so more quantitative binding studies are possible to confirm this hypothesis and show and quantifiy the three-way binding. One could think of FP, ITC, SPR, and it would be of real interest to know if there was cooperative binding or not.

d) Ideally the ubiquitination should be confirmed in vitro. This can be difficult though, and should probably not be a sticking point; but it would certainly be a strong point to confirm this hypothesis to see a rigorous comparison of CUL2ZYG11B in presence and absence of ORF10. In the absence of this a point mutant in the interaction would be really interesting, e.g. the one used in the Gygi/Elledge analysis. Identification and analysis of a point mutant that interferes with E2 binding would then further strengthen the analysis.

e) The TMT analysis is performed on multiple cell types but only one vulcanoplot is shown. Also the confirmed proteins are not shown on this plot. The analysis needs to be more thorough and explained in more detail. Deposition of results and tables as supplementary data are required. It would be good to colour the plot by go-terms used in the analysis.

f) The authors jump from analyzing a series of proteins to a single protein (IFT46) that is then expected to drive the effects on cilia in a manner that I cannot follow. If this one protein is expected to be that important for the observed effect it should be studied in more detail.

g) The mouse experiments with just spike and orf10 are interesting, but I can not judge if this is a meaningful experiment. The same holds for the cilia- experiments which are all done on 5 cells only, I cannot tell if that is a reasonable number, it seems rather low to an outsider

h) Discussion should include the E3 redirection literature, e.g. thalidomide/lenolidomide etc.

i) Also introduction and discussion need a more extensive discussion of the known literature on ZYG11B known targets /degrons to place the new findings in perspective

Reviewer #2 (Comments to the Authors (Required)):

Wang et al. used cell lines and mice to study the role of ORF10 of SARS-CoV-2. They find that ORF10 protein physically interacts with the CUL2(ZYG11B) complex and increases its E3 ubiquitin ligase activity. They show that increased E3 activity leads to enhanced proteasomal degradation of IFT46 (IFT B complex component), which leads to reduced ciliogenesis. They use hACE2 transgenic mice to show that in vivo exposure to SARS-CoV-2 ORF10 alone (using S protein pseudotyped lentivirus for gene transfer) leads to damaged ciliated cells. They suggest that this mechanism explains the loss of possibly cilium-related COVID-19 disease symptoms (loss of smell, loss of taste). The main concern with the study is that it contradicts the findings of a previously published paper (Mena, 2021) - both in terms of the global protein level changes induced by ORF10 expression in the same cell (HEK293T) AND in the ability of ORF10 to impact proteasomal degradation - and the discrepancies between their data and Mena et al. are not addressed. The authors present an intriguing mechanism, but their cell culture and S-pseudotyped lenti transduced mouse airways are not likely to adequately model SARS-CoV-2 infection of multiciliated cells. Further, studies (Robinot, 2021; He, 2020) have shown that transcription factors (FOXJ1, RFX3) responsible for global expression of ciliated cell genes in multiciliated airway epithelial cells are downregulated after SARS-CoV-2 infection, in fact, ciliated cell transcripts are broadly downregulated. While the mechanism for this is not yet clear, it is a much more likely reason for loss of cilia or defective cilia than the mechanism put forth by the authors which may be targeting the degradation of only specific components. To validate their findings, the authors would need to reconcile their data with Mena et al. They should compare their mass spec results and demonstrate that ORF10 enhances proteasomal degradation via the CUL2(ZYG11B) complex using the assay used by Mena et al. Further, the authors would need to validate their findings on a motile, multiciliated cell culture model, ex. primary airway epithelial cultures derived from the hACE2 mice infected with SARS-CoV-2 and/or S-lenti ORF10.

Major issues:

1. Abstract - "Loss of smell and taste are symptoms of COVID19, and they are related to cilia dysfunction." This is not yet demonstrated, this sentence should be changed to ...., and they MAY BE related to..."
2. The authors should speculate why S-lenti expression of ORF10 leads lung damage ("interstitial congestion, epithelial damage, and peribronchiolar lymphocytic inflammation surrounding bronchioles"). Although S-pseudotyped, and thus should enter ciliated cells through hACE2, this is essentially the same gene transfer method that has been explored for gene therapy in humans. Overexpression of ORF10 also did not appear to cause toxicity or death in the cell culture models.
3. Fig 4A - HA WB for ORF10 shows no reactivity in lane 2 and only a faint band in lane 4. MG132 treatment seems to lead to ORF10 accumulation - does this mean that ORF10 is proteasomally degraded? How does it impact IFT46 protein degradation? Band intensities are not always reflective of the values indicated.
4. Fig 4G - the label says ORF10-HA, but it appears that ORF10-HA was not part of the experiment.
5. Fig 5B - mouse bronchioles do not contain Krt5 expressing basal cells
6. Fig 6 - there are no methods describing SARS-CoV-2 infection of mice

Minor issues
1. Cilium = singular, cilia = plural - this should be corrected throughout the manuscript
2. Introduction - correct "respiratory distress syndrome" to acute respiratory distress syndrome (ARDS).

Reviewer #3 (Comments to the Authors (Required)):

Liying Wang et al. report that the SARS-CoV-2 ORF10 protein increases the activity of the CUL2-ZYG11B ubiquitin ligase complex, resulting in the increased degradation of ciliary proteins. First, the authors confirm the previously reported ORF10-ZYG11B interaction, and then demonstrate that this interaction increases the E3 ligase activity of the CUL2-ZYG11B complex, using a reconstituted in vitro ubiquitination system. Through a series of co-immunoprecipitation experiments, they also reveal an interaction between ORF10 and UBE2D1, leading to a model where ORF10 would link the E3 enzyme CUL2-ZYG11B and the E2 enzyme UBE2D1, resulting in an enhanced ubiquitination activity of the complex. Through a quantitative proteomic analysis of 293T cells expressing or not ORF10, the authors then show that ORF10 induces a marked decrease in multiple proteins involved in ciliogenesis, including the intraflagellar transport protein IFT46. Consistently, they show that ORF10 transfection inhibits primary cilium formation and maintenance in the MRC5 and NIH-3T3 cell lines. This inhibition is partially reverted by IFT46 overexpression, implicating the decrease in this intraflagellar transport protein in the process of cilia loss. To evaluate the role of ORF10 in vivo, the authors treated hACE2-knock-in mice with lentivectors expressing the SARS-CoV-2 spike +/- OF10. These experiments yielded images suggesting that intranasal administration of the ORF10+ lentivector induces lung pathology and motile cilia loss in the airways of treated mice.

Main comment:
The study is original and important, as it provides the first mechanistic model for the loss of airway motile cilia characteristic of SARS-CoV-2 infection. The findings are clinically relevant, as cilia loss may play a role in the efficient propagation of SARS-CoV-2 in the respiratory tree, and may also promote bacterial superinfections. The biochemistry, proteomic, and cell biology data are overall convincing. In contrast, the animal model experiments are performed on an unspecified number of animals, with results that are not quantified nor repeated. The in vivo findings are not entirely expected, as it is not clear why expression of ORF10 alone would induce lung pathology with interstitial congestion and lymphocytic infiltration. Therefore, the in vivo data should be strengthened by repeated experiments with clearly quantified outcomes.

Specific points:
- It is surprising that an ORF10 lentivector pseudotyped with the SARS-CoV-2 spike could infect NIH-3T3 cells, which do not express the human ACE2 receptor (Fig. S7). Did the authors used a variant spike that can mediate entry in mouse cells? If so, this should be specified in the Methods section. The lentivector used in these and in the in vivo experiments are insufficiently described.

- The meaning of the schematics in Fig. 5A is not clear, and is not explained in a legend. The dark pink lung symbols in rows 2 and 3 would suggest that both ORF10-negative and ORF10-positive lentivectors induced lung infiltration and congestion. Is this what the authors mean? If so, this would imply an effect of the spike alone, which is not discussed at all in the Results section. The use of two SARS-CoV-2 proteins complexifies the interpretation of the data.

- In panel 5B, it is not clear why the authors chose to use a basal cell marker to label bronchiolar tissue, rather than a ciliated cell marker that would have been more relevant to their study.

- The meaning of the histology images shown in Fig. 5D is unclear. It is difficult to see whether there is more congestion and lymphocytic infiltration in the ORF10+ than in the ORF10- condition. The explanations for the red arrows and dashed lines are missing.

- As stated above, none of the in vivo data shown in Fig. 5 and 6 are quantified, and the authors provide no evaluation of the reproducibility of their findings.

- The authors provide remarkable proteomics data on ORF10-expressing 293-T cells, with a massive downregulation of proteins (352 downregulated and only 2 upregulated), involving primarily components and regulators of motile cilia. They mention in the discussion the study of Mena et al. (PNAS 2021), where a similar proteomic approach (TMT labeling followed by MS) in ORF10 expressing 293-T cells yielded very different results. Mena et al. reported fewer modulated proteins (8 downregulated and 91 upregulated), and no preferential loss of ciliary proteins. The authors should discuss the reasons for these discrepancies, as the downregulation of ciliary components is central to their message.

- It is not clear if the 293-T cells used for the proteomic analysis were transfected or transduced with a lentivector to induce ORF10 expression. Also, the authors mention in line 164 that the analysis was carried out across all replicates, but do not mention the number of replicates, nor the reproducibility of their findings when comparing different replicates. These points

should be specified.

- The authors compared their set of proteins modulated by ORF10 to the set of proteins modulated by SARS-CoV-2 infection from the study of Bojkova et al. They observe a 22% overlap between the two datasets and mention in line 170 that most of the co-modulated proteins were reduced upon SARS-CoV-2 infection (Fig. S3B, C). This reduction is far from obvious visually (Fig. S3C), and the two datasets showed a trend for consistent variation at only one of the 4 time points tested (Fig. S3B). The overlap between the two datasets should be discussed in a more balanced fashion.

- The statement on lines 218-220 that MG132 partially rescued ORF10-mediated promotion of protein degradation while 3-MA did not is questionable, as bands in lanes 3 and 4 of Fig. 4A look quite similar (except for ORF10 itself). The authors should repeat these experiments and show with statistics whether the differences between MG132 and 3-MA treatment are significant.

- The subcellular distribution of ORF10 is not precisely documented. In Figs. 3 and 4., what are the subcellular compartments involved? Also, could ORF10 be seen to colocalize with primary cilia before inducing their disappearance?

- If feasible, it would be informative to analyze ORF10 localization in primary multicilated cells, which are the actual target cells of SARS-CoV-2. Could the authors document the localization of ORF10 in multicilated cells at an early stage of infection, prior to the loss of cilia?

Minor points
- The affiliations are not numbered by order of appearance.
- The reference format is not always consistent. Some of the references end with a + sign, like the first one.
- Abbreviations are not always defined. For instance, Ac-Tubulin is to be defined in line 193.
- Explain that numbers below Western blot bands represent quantification of those bands.
- Probable mistake in the legend of Fig. 4G (replace ORF10 by ZYG11B). Minus signs are missing in the legend of Fig. 4E.
- The manuscript would benefit from English editing. For instance, the word "for" is missing in line 141, and the word "cell" is missing in line 211.

Point by point responses:

We thank the three experts for their time in carefully reviewing our work, and we are grateful for the excellent comments and guidance about how to improve our study. Over the last five months, we have undertaken a series of additional experiments guided by the reviewers' suggestions, and have now prepared a revised manuscript that describes the newly acquired results. We trust that our new findings, our reworked manuscript, and our point-by-point responses (below) together address the reviewers' comments. In our view, the review process has worked as designed, and our revised study is substantially improved in terms of its technical rigor and scientific implications.

Reviewer #1 (Comments to the Authors (Required)):

In this manuscript the authors study the previously identified interaction between SARS-COV-2 ORF10 and the ubiquitin E3 ligase adapter ZYG11B. They confirm the interaction by pull-down assays and find that it extends to the E2 enzyme UBE2D1. They then test for protein changes upon ORF10 expression using TMT mass spectrometry and identify a series of proteins involved in cilia biogenesis and maintenance that have reduced abundance after ORF10 expression. After confirming this for a small subset they focus on one protein, IFT146 where they show more detailed effects of ubiquitination. They then study cilia formation in the presence of ORF10 on 5 cells and see that ZYB11b is important for this. Finally, they analyze the cilia in mice that are infected with a spike and ORF10 containing lentivirus, where again they observe an effect on cilia.

A new example of an E3-ligase adapter being hijacked to a set of new targets is of general interest scientifically and if this underlies the loss of smell and taste observed in COVID-19 it would have a very broad impact. However, exactly because of its potential implications it is important to

solidify the data. At this point there are a number of concerns and aspects that require better controls and more solid data.

a) A major concern is that an earlier paper by Steve Elledge and with involvement from the Gygi lab did a similar TMT proteomics experiment, also on 293T cells, but did not observe the differences in cilia-related proteins mentioned here. The authors do mention this, but seem to treat it rather lightly, whereas I find it a real concern, as the Gygi lab has very well-controlled proteomics, that I would trust over most others in the world. It would be good to provide a more detailed comparison with the outcome of the Gygi/Elledge experiment to see if there is an explanation for this difference. One could imagine that the levels of ORF10 are important, but then it would be good to analyze this carefully.

Response: First, we would like to thank Reviewer for the thorough and highly insightful review, and for the helpful comments. With this first comment, the Reviewer provides a very thought-provoking suggestion: that the levels of ORF10 might cause the observed difference in the MS results between our and Mena *et al.*'s results (Mena et al., 2021). Pursuing this idea, we have now measured the protein levels of these cilium-related proteins in a set of cell samples with a gradient of increasing ORF10 expression. We found that the protein level of these cilium-related proteins was dramatically decreased as ORF10 expression increased (Fig. S2J, K), indeed supporting that the protein level of ORF10 does affect the stability of these cilium-related proteins.

Considering that Mena *et al.*'s results did not include detected expression for ORF10, we explored whether the detected differences in cilium-related protein levels between the two studies may reflect the difference in the ORF10 protein levels. We first monitored the expression levels of ORF10 by transfecting HEK293T cells with pcDNA-ORF10-HA plasmids or pHAGE-ORF10-HA plasmids, the plasmids respectively used in our study and Mena *et al.* study.

We found ORF10-HA was successfully detected in cells harboring the pcDNA-ORF10-HA plasmid, but not in cells harboring pHAGE-ORF10-HA plasmids (Fig. S2I). Moreover, we found the protein levels of the cilium-related proteins was dramatically decreased in HEK293T cells transfected with pcDNA-ORF10-HA, but not pHAGE-ORF10-HA (Fig. S2L, M), indicating that the ORF10 level could account for the observed differences in cilia-related protein levels between the two studies.

b) The difference extends to the fact that the Gygi/Elledge analysis also did not observe a difference in the normal function of ZYG11B, which would also contradict a wholesale redirection of the targeting of ZYG11B. If the authors suggest here a new series of targets for ZYG11B, it would be good to show how ZYG11B affects its usual targets under these conditions. Or do the ciliated proteins have the ZYG11B degrons?

Response: We thank the Reviewer for this insightful comment. To test whether altering the ORF10 protein level affects the difference in the normal function of ZYG11B, we reconstituted a Global Protein Stability (GPS) system with reporters to detect $CUL2^{ZYG11B}$ activity (*Nota Bene*: this is the experimental setup used in the Mena *et al.* study). We found that HEK293T cells transfected with pcDNA-ORF10-HA had a reduced GFP:DsRed ratio value compared to the empty vector control cells, which was similar with the effect of cells overexpressing ZYG11B (Fig. S2N). Therefore, the high expression level of ORF10 may enhance the reported function of ZYG11B.

Given its lack of ZYG11B degrons (Fig. S4I), IFT46 may interact with ZYG11B through some other mechanism(s). Pursuing this, we mutated three residues of ZYG11B (ZYG11B-W522A, D526A, N567A; hereafter referred to ZYG11B-Mut) known as essential for the recognition of Gly/N-degron sequences in its substrates (Yan et al., 2021), and detected the interaction between ZYG11B-Mut and IFT46 in HEK293T cells. Co-IP showed no difference in the extent of the ZYG11B-IFT46 interaction between WT ZYG11B and the ZYG11B variant incapable of

recognizing Gly/N-degron sequences (Fig. S4J). These results suggest that the interaction of ZYG11B and IFT46 is not based on a Gly/N-degron-based mechanism.

To further analyze the relationship between ZYG11B and IFT46, we generated a series of internal deletion mutants of IFT46 to map the binding regions for ZYG11B (Fig. 5A). We found that the C-terminal domain (amino acids 182–288, IFT46-C2) of IFT46 was sufficient for binding to ZYG11B (Fig. 5B, C). Further, absence of the interaction between IFT46 and ZYG11B perturbed the effect of ZYG11B overexpression on the ubiquitination of IFT46 (Fig. 5D). The depletion of the C2 region of IFT46 (FT46-$^{\triangle}$C2) also blocked the degradation of IFT46 after ORF10 expression (Fig. 5E, F). In addition, IFT46-$^{\triangle}$C2 expression rescued the ciliogenesis defects in ORF10-expressing cells (Fig. 5I, J). These results show that $CUL2^{ZYG11B}$ promoting IFT46 degradation to impair cilium biogenesis and maintenance via a non-classical way, which recognized the C2 domain of IFT46.

c) The authors propose a three-way interaction between ORF10, ZYG11B and UBE2D1, but only show individual pull-downs. These are small peptides or easily produced proteins (see e.g., the recent structural data on ZYG11b), so more quantitative binding studies are possible to confirm this hypothesis and show and quantify the three-way binding. One could think of FP, ITC, SPR, and it would be of real interest to know if there was cooperative binding or not.

Response: Thank you for the insightful comment. To assess the three-way interaction between ORF10, ZYG11B, and UBE2D1, we performed FLAG-pulldown analysis of ORF10-FLAG with adding ZYG11B and UBE2D1: these assays showed that ORF10 can simultaneously bind to both ZYG11B and UBE2D1 (Fig. 1I lane 2, 1J lane2). To quantify the three-way binding, we used SPR to quantify the binding affinity for the ORF10-ZYG11B interaction and ORF10-UBE2D1 interaction. The detected binding affinity for the ORF10-ZYG11B interaction (0.45nM) was stronger than for the ORF10-UBE2D1 interaction (33.74nM) (Fig. 1H). We also performed

competitive binding experiments with these three proteins. Briefly, as the ZYG11B concentration increased in our gradient sample series, ZYG11B outcompeted UBE2D1 for binding to ORF10 (Fig. 1I). However, increasing the UBE2D1 concentration had little effect on the interaction between ZYG11B and ORF10 (Fig. 1J). Thus, SPR, FLAG-pulldown, and binding competition assays support that ORF10 can simultaneously directly bind to ZYG11B and to UBE2D1, although clearly ZYG11B has relatively higher binding affinity for ORF10 compared to UBE2D1.

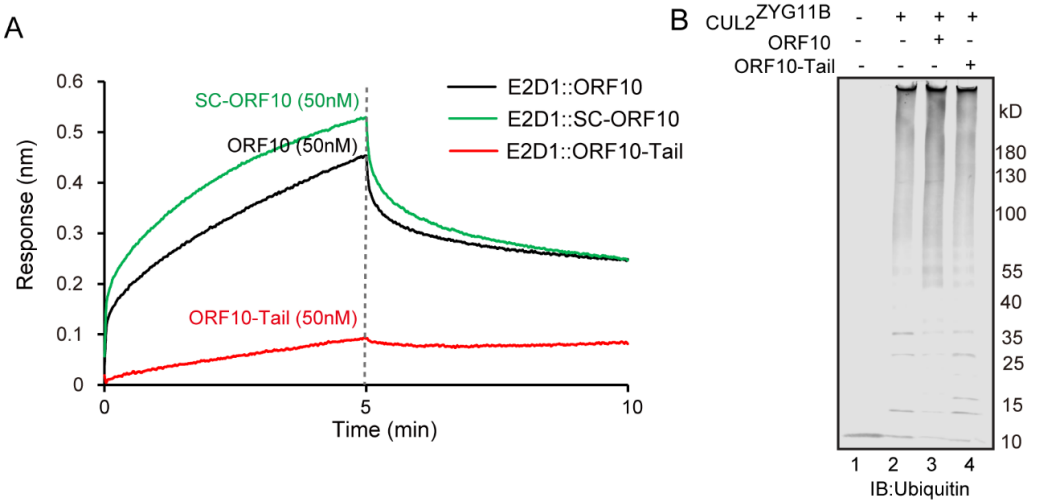
d) Ideally the ubiquitination should be confirmed in vitro. This can be difficult though, and should probably not be a sticking point; but it would certainly be a strong point to confirm this hypothesis to see a rigorous comparison of CUL2ZYG11B in presence and absence of ORF10. In the absence of this a point mutant in the interaction would be really interesting, e.g. the one used in the Gygi/Elledge analysis. Identification and analysis of a point mutant that interferes with E2 binding would then further strengthen the analysis.

Response: We thank the Reviewer for this very thought-provoking suggestion. As the Reviewer points out, it can be difficult to reconstitute a $CUL2^{ZYG11B}$ *in vitro* ubiquitination system; we therefore used the $CUL2^{ZYG11B}$ immunocomplex to assess $CUL2^{ZYG11B}$-mediated ubiquitination (Fig. 1B). We found that ORF10 greatly enhanced the E3 ligase activity of the $CUL2^{ZYG11B}$ immunocomplex (Fig. 1B and Fig. S1R, S). And SC-ORF10, which could not interact with ZYG11B (Fig. 1D), did not affect the E3 ligase activity of $CUL2^{ZYG11B}$ (Fig. 1E). Therefore, the ZYG11B-ORF10 interaction is essential for the effect of ORF10 in $CUL2^{ZYG11B}$ E3 ligase activity.

Given our results from GST-Pulldown and SPR study showing that both ORF10 and SC-ORF10 can directly bind to UBE2D1 (Fig. S1J, R), and considering that SC-ORF10 has 13 fewer amino acids than ORF10, we speculated that the 13 aa tail of ORF10 may not bind to UBE2D1.

Pursuing this, we synthesized the 13 aa ORF10 tail and performed SPR: the 13 aa ORF10 tail had lower response value for UBE2D1 compared to ORF10 and compared to SC-ORF10 (Fig. R1A); further, the13 aa ORF10 tail could not stimulate the E3 ligase activity of the $CUL2^{ZYG11B}$ (Fig. R1B). Together, our results reveal that the interaction in both ORF10-ZYG11B and ORF10-UBE2D1 are essential for the effect of ORF10 on $CUL2^{ZYG11B}$ E3 ligase activity.

Figure R1



To confirm an impact from ORF10 on $CUL2^{ZYG11B}$ substrate ubiquitination, we examined the IFT46 ubiquitination level upon transfection of HEK293T cells with pcDNA-ORF10-HA. Cells expressing ORF10 significantly increased the extent of IFT46 ubiquitination compared to empty vector control cells (Fig. 4J). Moreover, there was no difference in the extent of IFT46 ubiquitination between cells expressing SC-ORF10 and control cells (Fig. 4J). These findings are consistent with the detected impacts of ORF10 on the E3 ligase activity of $CUL2^{ZYG11B}$ (Fig. 1E), and reveal that ORF10 could also enhanced the ubiquitination level on $CUL2^{ZYG11B}$ substrate.

To explore the effect of ZYG11B-IFT46 interaction on the ubiquitination of IFT46, we mapped the binding regions for ZYG11B in IFT46, and found the C-terminal domain (amino acids 182–288, IFT46-C2) of IFT46 was essential for binding to ZYG11B (Fig. 5B, C). Furthermore,

absence of the interaction between IFT46 and ZYG11B indeed perturbed the effect of ZYG11B overexpression on the ubiquitination of IFT46 (Fig. 5D). The depletion of the C2 region of IFT 46 also blocked the degradation of IFT46 after ORF10 expression (Fig. 5E, F). Therefore, the interaction between ZYG11B and IFT46 is necessary for the effect of ORF10 expression on the $CUL2^{ZYG11B}$ substrate.

e) The TMT analysis is performed on multiple cell types but only one volcano plot is shown. Also, the confirmed proteins are not shown on this plot. The analysis needs to be more thorough and explained in more detail. Deposition of results and tables as supplementary data are required. It would be good to colour the plot by go-terms used in the analysis.

Response: Thank you for focusing our attention on these omissions. We have substantially revised the relevant results and M&M section to provide these details. To clarify, we here conducted 6-plex TMT-based proteomics analysis, seeking to profile changes in protein levels between ORF10-expressing and mock-transfected HEK293T cells. Two groups of samples, each containing three replicates, were compared in our study. The names of confirmed proteins have been added on the volcano plot (Figure 2A). As suggested by the reviewer, we have also:

—added a supplementary table of all the proteins we identified in the TMT analysis (Table S1);

—added a supplementary table of all GO-terms significant enriched among the DEPs (Table S2);

—colored the GO-terms in Table S2 according to the Fig. 2B.

—deposited the mass spectrometry proteomics data to the ProteomeXchange Consortium via the iProX partner repository with the dataset identifier PXD031842.

f) The authors jump from analyzing a series of proteins to a single protein (IFT46) that is then expected to drive the effects on cilia in a manner that I cannot follow. If this one protein is expected to be that important for the observed effect it should be studied in more detail.

Response: Sorry for the lack of clarity here; in the revised manuscript, we have now explicated the sequence of results that lead us to IFT46. To clarify: we first examined proteins known to be required for cilia biogenesis (TTBK2, BBS4, SEPTIN2, TALPID3, and IFT46) (Fig. 2D, E). We found that the presence of ORF10 led to ubiquitin-proteasome mediated reductions in the levels of all these proteins except TTBK2 (Fig. 4A, B). Subsequently, cycloheximide (CHX) chase assays showed that ORF10 strongly promoted IFT46 degradation compared with the control group; less pronounced impacts from ORF10 were observed on the extent of TALPID3, BBS4, and SEPTIN2 degradation (Fig. S4A-F). These findings narrowed our focus to the required cilia biogenesis protein IFT46, and we found that ORF10 facilitates IFT46 ubiquitination by stimulating the ubiquitination activity of $CUL2^{ZYG11B}$ (Fig. 4F).

g) The mouse experiments with just spike and orf10 are interesting, but I cannot judge if this is a meaningful experiment.

Response: Thank you for focusing our attention here. To further explored potential impacts of ORF10 on the pathobiology of SARS-CoV-2 infection, we developed a direct way to examine the pathogenic effect(s) of ORF10 on ciliogenesis due to lacking a direct way to examine the pathogenic effect(s) of ORF10 in humans. Briefly, we generated the lentiviral pseudotype particles with expressing SARS-CoV-2 Spike, and then exposed hACE2 transgenic mouse to these particles (Fig. 6A). Given that the Spike glycoprotein from corona viruses facilitates binding to ACE2 (Walls et al., 2020), and the lentivirus pseudotyped particles do not contain other SARS-CoV-2 ORFs except for ORF10, Spike-lentivirus-ORF10 could mimic SARS-CoV-2 infection and specifically deliver the gene ORF10 to the host cells and exclude the effect of other SARS-CoV-2 ORFs on ciliogenesis.

The same holds for the cilia- experiments which are all done on 5 cells only, I cannot tell if that is

a reasonable number, it seems rather low to an outsider

Response: We have now completed a new set of experiments in which we quantified the proportion of ciliated epithelial cells in more than 300 epithelial cells per group (across five independent experiments) (Fig. 7E). A ciliated epithelial cells rate of 63.60% ± 1.806 was observed among the epithelial cells exposed to Spike-lentivirus-ORF10, compared to the 91.40% ± 1.030 and 91.80% ± 1.530 ciliated epithelial cells rate observed among the Spike-lentivirus group and MOCK group respectively (Fig. 7F). Moreover, we have also constructed a motile, multiciliated cell culture model to further confirm the effect of ORF10 on cilia. The multiciliated primary human nasal epithelial cells (HNECs) exposed to Spike-lentivirus-ORF10 displayed a significant reduction in the proportion of ciliated cells compared to the Spike-lentivirus and MOCK controls (Fig. 9A, B), thus confirming an impact of ORF10 on cilium biogenesis and maintenance.

h) Discussion should include the E3 redirection literature, e.g., thalidomide/lenolidomide etc.

Response: Thank you for these suggestions; we have added content in the discussion as follows:

"*Thalidomide and its derivatives lenalidomide are effective treatments for multiple myeloma; these agents inhibit cereblon (CRBN), a part of the cullin-4 E3 ubiquitin ligase complex CUL4-RBX1-DDB1(Fischer et al., 2014; Singhal et al., 1999). Inhibiting CUL2^{ZYG11B} using drugs and/or supplementing IFT46 (or other cilia related proteins) might be useful in fighting this coronavirus*."

i) Also, introduction and discussion need a more extensive discussion of the known literature on ZYG11B known targets /degrons to place the new findings in perspective

Response: Thank you for this guidance.

Our revised manuscript presents this content as follows: (lines 92–96), (lines 428–433)

INTRODUCTION

"*Cullin 2-RING (CUL2) is a ubiquitin E3 ligase comprising Cullin-2, RBX1, Elongin B, Elongin C, and ZYG11B. CUL2^{ZYG11B} regulates protein degradation by targeting the N-terminal glycine of substrate proteins. ZYG11B is the substrate receptor of CUL2 and responsible for recognition of the N-terminal glycine degrons (Eldeeb et al., 2019; Timms et al., 2019)*"

DISCUSSION

"*Recently, Yan et al. (2021) reported that three residues (W522, D526, N567) of ZYG11B are required for specific recognition of the Gly/N-degron by CRL2^{ZYG11B} (Yan et al., 2021). We found that the mutation of key recognition elements in ZYG11B did not affect the interaction of ZYG11B and IFT46 (Fig. S4J). CUL2^{ZYG11B}'s interaction with the C2 domain of IFT46 promotes IFT46 ubiquitination (Fig. 4B, C). Therefore, the interaction of ZYG11B with IFT46 was independent on its recognition of the Gly/N-degron.*"

We would again like to thank the Reviewer for the helpful guidance about how to improve our study.

Reviewer #2 (Comments to the Authors (Required)):

Wang et al. used cell lines and mice to study the role of ORF10 of SARS-CoV-2. They find that ORF10 protein physically interacts with the CUL2(ZYG11B) complex and increases its E3 ubiquitin ligase activity. They show that increased E3 activity leads to enhanced proteasomal degradation of IFT46 (IFT B complex component), which leads to reduced ciliogenesis. They use hACE2 transgenic mice to show that in vivo exposure to SARS-CoV-2 ORF10 alone (using S protein pseudotyped lentivirus for gene transfer) leads to damaged ciliated cells. They suggest that this mechanism explains the loss of possibly cilium-related COVID-19 disease symptoms (loss of smell, loss of taste).

The main concern with the study is that it contradicts the findings of a previously published paper (Mena, 2021) - both in terms of the global protein level changes induced by ORF10 expression in the same cell (HEK293T) AND in the ability of ORF10 to impact proteasomal degradation - and the discrepancies between their data and Mena et al. are not addressed.

Response: First, we would first like to thank Reviewer 2 for the very helpful comments and guidance to improve our study. To address the cause of the difference between our MS data and that of Mena *et al.*'s results (Mena et al., 2021), we carefully compared with the materials used in our and Mena *et al.*'s results. We found the ORF10 expression plasmid used in our study was different with that of Mena *et al.*'s results, and Mena *et al.*'s results did not include detected expression for ORF10. So we speculated that the protein level of ORF10 might be response for the difference between our and Mena *et al.*'s results. We first monitored the expression levels of ORF10 by transfecting HEK293T cells with pcDNA-ORF10-HA plasmids or pHAGE-ORF10-HA plasmids, the plasmids respectively used in our study and Mena *et al.*'s results. We found ORF10-HA was successfully detected in cells harboring the pcDNA-ORF10-HA plasmid, but not in cells harboring pHAGE-ORF10-HA plasmids (Fig. S2I). Given that the major difference between our and Mena *et al.*'s results focused on the cilium-related proteins, we have now measured the protein levels of these cilium-related proteins in a set of cell samples with a gradient of increasing ORF10 expression. We found that the protein level of these cilium-related proteins was dramatically decreased as ORF10 expression increased (Fig. S2J, K). Moreover, we found the protein levels of the cilium-related proteins was dramatically decreased in the cells transfected with pcDNA-ORF10-HA (Fig. S2M, N), indicating that the ORF10 level could account for the observed differences in cilia-related protein levels between the two studies.

The authors present an intriguing mechanism, but their cell culture and S-pseudotyped lenti

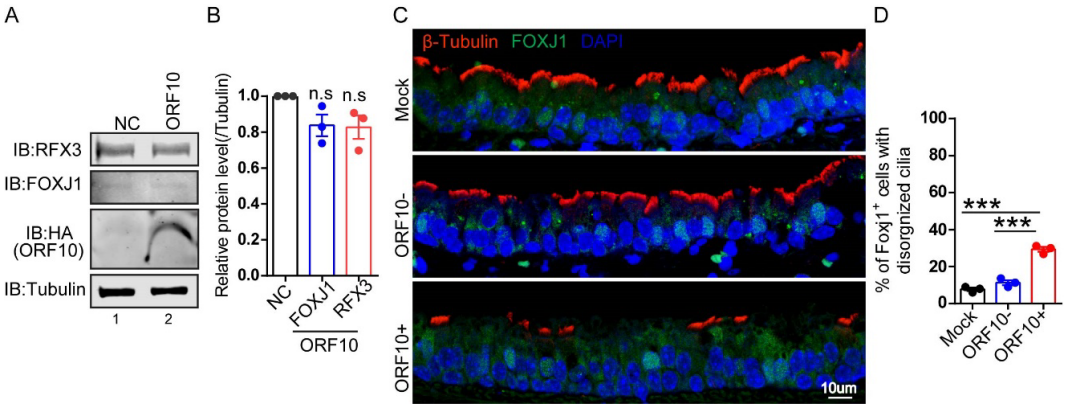
transduced mouse airways are not likely to adequately model SARS-CoV-2 infection of multiciliated cells.

Response: We thank Reviewer 2 for this very thought-provoking suggestion. We have constructed a human multiciliated cell culture model to validate our main findings. As is shown in Fig. 9A, we infected primary human nasal epithelial cells (HNECs) with Spike-lentivirus-ORF10. The multiciliated HNECs exposed to Spike-lentivirus-ORF10 displayed a significant reduction in the proportion of ciliated cells compared to Spike-lentivirus and MOCK group (Fig. 9B). This finding is similar to cilia loss observed for SARS-CoV-2 infection in the Robinot *et al*. study (Robinot et al., 2021). These results confirmed an impact of ORF10 in perturbation of cilium biogenesis and maintenance.

Further, studies (Robinot, 2021; He, 2020) have shown that transcription factors (FOXJ1, RFX3) responsible for global expression of ciliated cell genes in multiciliated airway epithelial cells are downregulated after SARS-CoV-2 infection, in fact, ciliated cell transcripts are broadly downregulated. While the mechanism for this is not yet clear, it is a much more likely reason for loss of cilia or defective cilia than the mechanism put forth by the authors which may be targeting the degradation of only specific components.

Response: We thank Reviewer 2 for pointing out this problem. Although we could not exclude an effect from downregulated transcription factors (FOXJ1, RFX3) on cilia dysfunction after SARS-CoV-2 infection, we did find that the transfection of ORF10 in HEK293T cells have little effect on the expression of FOXJ1 and RFX3 (Fig. R2A, B), and FOXJ1 could still be detected in cilia damaged epithelial cells with ORF10 expression (Fig. R2C, D). Therefore, there may be complex pathological mechanisms connecting COVID-19 symptoms with cilia dysfunction, we have toned down several assertions and have added discussion content related to this point.

Figure R2



To validate their findings, the authors would need to reconcile their data with Mena et al. They should compare their mass spec results and demonstrate that ORF10 enhances proteasomal degradation via the CUL2(ZYG11B) complex using the assay used by Mena et al.

Response: Thank you for your insightful suggestion. First, we performed a more detailed comparison between our proteomic data and those by Mena *et al*., including proteomic results and experimental designs. The identified proteins in HEK293T cells are very similar between our and Mena *et al*.'s data (Fig. S2G), but there is little overlap between differentially expressed proteins (DEPs) in our and Mena *et al*.'s data (Fig. S2H). As described above, the protein level of ORF10 does affect the stability of these cilium-related proteins. To confirm an impact of the ORF10 protein level on the proteasomal degradation via the $CUL2^{ZYG11B}$ complex, we reconstituted the GPS assay used in Mena *et al*. study. We found the GFP:DsRed ratio is decreased after pcDNA-ORF10-HA expression used in our work, which was similar with the effect of ZYG11B overexpression (Fig. S2N). Therefore, the protein level of ORF10 may account for the difference between our results and those of Mena *et al*.

Further, the authors would need to validate their findings on a motile, multiciliated cell culture model, ex. primary airway epithelial cultures derived from the hACE2 mice infected with SARS-CoV-2 and/or S-lenti ORF10.

Response: Thank you for your suggestions. We have constructed a human multiciliated cell culture model and performed infection assays using Spike-lentivirus-ORF10. The multiciliated primary human nasal epithelial cells (HNECs) exposed to Spike-lentivirus-ORF10 displayed a significant reduction in the proportion of ciliated cells compared to the Spike-lentivirus and MOCK controls (Fig. 9A, B). This finding is similar to cilia loss observed for SARS-CoV-2 infection in the Robinot *et al.*'s study (Robinot et al., 2021). These results confirmed an impact of ORF10 in perturbation of cilium biogenesis and maintenance.

Major issues:

1. Abstract - "Loss of smell and taste are symptoms of COVID19, and they are related to cilia dysfunction." This is not yet demonstrated, this sentence should be changed to ...., and they MAY BE related to..."

Response: Thanks for this guidance. we have corrected it. Line 43: "are" should be "may be".

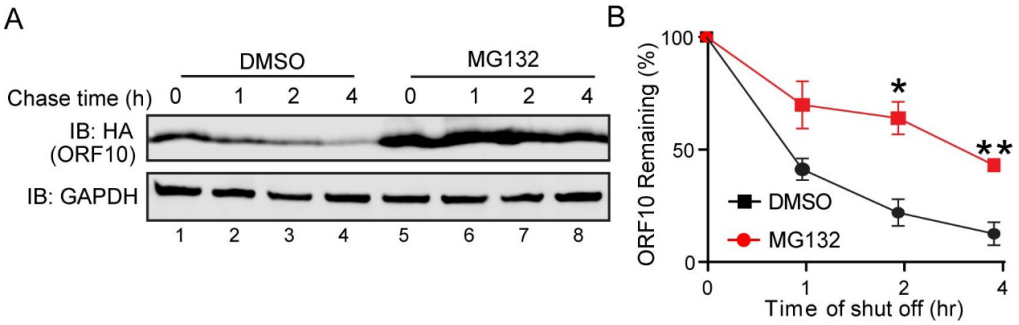
2. The authors should speculate why S-lenti expression of ORF10 leads lung damage ("interstitial congestion, epithelial damage, and peribronchiolar lymphocytic inflammation surrounding bronchioles"). Although S-pseudotyped, and thus should enter ciliated cells through hACE2, this is essentially the same gene transfer method that has been explored for gene therapy in humans. Overexpression of ORF10 also did not appear to cause toxicity or death in the cell culture models.

Response: Thanks for pointing out this problem. GO analyses indicated that the set of proteins with reduced accumulation in the ORF10 expressing cells showed enrichment for functional annotations related to the terms "cell cycle" and "RNA polymerase II transcription factor complex" except ciliogenesis (Fig. 2B). It may be count for why S-lenti expression of ORF10 leads lung damage. Thus, ORF10 may play multiple roles in the pathology of SARS-CoV-2, in this study, we mainly focused on the role of ORF10 during ciliogenesis.

3. Fig 4A - HA WB for ORF10 shows no reactivity in lane 2 and only a faint band in lane 4. MG132 treatment seems to lead to ORF10 accumulation - does this mean that ORF10 is proteasomal degraded? How does it impact IFT46 protein degradation? Band intensities are not always reflective of the values indicated.

Response: Thanks for pointing out this problem. We have repeated and quantified the experiment. ORF10 could downregulate some cilium-related protein through ubiquitin-proteasome pathway excluding TTBK2 (Fig. 4A, B). MG132 treatment apparently leads to ORF10 accumulation. We investigated this further by performing cycloheximide (CHX) chase assays: adding MG132 resulted in strong accumulation of ORF10 (Fig. R3). Therefore, ORF10 may be degraded through proteasomes. Although ORF10 can apparently be degraded through proteasomes, the retained ORF10 was sufficient to promote IFT46 degradation in HEK293T cells after treatment with cycloheximide (Fig. 4C, D).

Figure R3



4. Fig 4G - the label says ORF10-HA, but it appears that ORF10-HA was not part of the experiment.

Response: Sorry for this mistake; we have corrected it. Fig. 4H: “ORF10-HA” should be “ZYG11B-FLAG”.

5. Fig 5B - mouse bronchioles do not contain Krt5 expressing basal cells

Response: Sorry for this mistake, Line 381: “bronchioles” should be “trachea”.

6. Fig 6 - there are no methods describing SARS-CoV-2 infection of mice

Response: Thanks for your helpful guidance here! We have added it.

Our revised manuscript presents this content as follows: (lines 590–596)

“*Mouse Challenge Experiments*

For intranasal infection, hACE2 mice were anesthetized with sodium pentobarbital at a dose of 50 mg/kg though intraperitoneal route, and then intranasally infected with 10^5 *pfu of SARS-CoV-2. Mice were sacrificed on day 4 post infection for tissue processing.*

Animals were housed in an isolator in BioSafety Level 3 animal facilities at the Institute of Microbiology, Chinese Academy of Sciences. The organs and fluids recovered from the animals infected with live SARS-CoV-2 following the approved standard operating procedures of these facilities.”

Minor issues

1. Cilium = singular, cilia = plural - this should be corrected throughout the manuscript

Response: Sorry for this mistake, we have corrected it throughout the manuscript.

2. Introduction - correct "respiratory distress syndrome" to acute respiratory distress syndrome (ARDS).

Response: Thanks for this guidance. We have corrected it. Line 61-62: “respiratory distress syndrome” should be “acute respiratory distress syndrome (ARDS)”.

We would like to take this opportunity to express our gratitude for the helpful guidance. Many thanks!

Reviewer #3 (Comments to the Authors (Required)):

Liying Wang et al. report that the SARS-CoV-2 ORF10 protein increases the activity of the CUL2-ZYG11B ubiquitin ligase complex, resulting in the increased degradation of ciliary proteins. First, the authors confirm the previously reported ORF10-ZYG11B interaction, and then demonstrate that this interaction increases the E3 ligase activity of the CUL2-ZYG11B complex, using a reconstituted in vitro ubiquitination system. Through a series of co-immunoprecipitation experiments, they also reveal an interaction between ORF10 and UBE2D1, leading to a model where ORF10 would link the E3 enzyme CUL2-ZYG11B and the E2 enzyme UBE2D1, resulting in an enhanced ubiquitination activity of the complex. Through a quantitative proteomic analysis of 293T cells expressing or not ORF10, the authors then show that ORF10 induces a marked decrease in multiple proteins involved in ciliogenesis, including the intraflagellar transport protein IFT46. Consistently, they show that ORF10 transfection inhibits primary cilium formation and maintenance in the MRC5 and NIH-3T3 cell lines. This inhibition is partially reverted by IFT46 overexpression, implicating the decrease in this intraflagellar transport protein in the process of cilia loss. To evaluate the role of ORF10 in vivo, the authors treated hACE2-knock-in mice with lentivectors expressing the SARS-CoV-2 spike +/- OF10. These experiments yielded images suggesting that intranasal administration of the ORF10+ lentivector induces lung pathology and motile cilia loss in the airways of treated mice.

Main comment:

The study is original and important, as it provides the first mechanistic model for the loss of airway motile cilia characteristic of SARS-CoV-2 infection. The findings are clinically relevant, as cilia loss may play a role in the efficient propagation of SARS-CoV-2 in the respiratory tree, and may also promote bacterial superinfections. The biochemistry, proteomic, and cell biology data are overall convincing. In contrast, the animal model experiments are performed on an

unspecified number of animals, with results that are not quantified nor repeated. The in vivo findings are not entirely expected, as it is not clear why expression of ORF10 alone would induce lung pathology with interstitial congestion and lymphocytic infiltration. Therefore, the in vivo data should be strengthened by repeated experiments with clearly quantified outcomes.

Response: We thank the Reviewer for these helpful comments, all of which have been valuable as we revised our paper and improved our study. We would also like to note that comments have provided important guiding significance to our overall research program. We have studied comments carefully and thoroughly and revised our manuscript accordingly. Of particular note, we have repeated and quantified *in vivo* experiments (Fig. 7, Fig. 8).

Specific points:

- It is surprising that an ORF10 lentivector pseudo typed with the SARS-CoV-2 spike could infect NIH-3T3 cells, which do not express the human ACE2 receptor (Fig. S7). Did the authors used a variant spike that can mediate entry in mouse cells? If so, this should be specified in the Methods section. The lentivector used in these and in the in vivo experiments are insufficiently described.

Response: Thank you for pointing out the lack of essential methodological detail in our originally submitted manuscript. At 48h prior to virus infection, the NIH-3T3 cells were transfected with a plasmid for the expression of hACE2. Accordingly, ORF10 lentivector could infect NIH-3T3 cells. We have added it in methods.

Our revised manuscript presents this content as follows: (lines 541–552)

"Lentivirus production and transduction of NIH-3T3 cells

The lentiviral vectors containing cytomegalovirus (CMV) to drives the expression of ORF10 were used in this study. 293T cells were transfected with the target vector and the other two plasmids (pCAGGS-Spike, psPAX) and were incubated in fresh medium after six hours of transfection. Supernatants containing lentivirus were collected and stored at -80℃ after 24h, 48h and 72h of transfection. Lentivirus titers were identified by infecting 293T cells with viral supernatant for 24

h. The NIH-3T3 cells was transfected with pDC316-human ACE2 plasmids using Lipofectamine 2000 reagent according to the manufacturer's instructions for 48h. cells were transduced with 107 MV Lentiviral Vector Pseudotypes to induce the expression of ORF10. Cells were serum-starved for 24h to induce cilium formation. After cilium formation upon serum starvation for 24h, MRC-5 and NIH-3T3 cells were harvested at 2 days after transfection of Lentiviral Vector Pseudotypes for fluorescence microscopy."

- The meaning of the schematics in Fig. 5A is not clear, and is not explained in a legend. The dark pink lung symbols in rows 2 and 3 would suggest that both ORF10-negative and ORF10-positive lentivectors induced lung infiltration and congestion. Is this what the authors mean? If so, this would imply an effect of the spike alone, which is not discussed at all in the Results section. The use of two SARS-CoV-2 proteins complexifies the interpretation of the data.

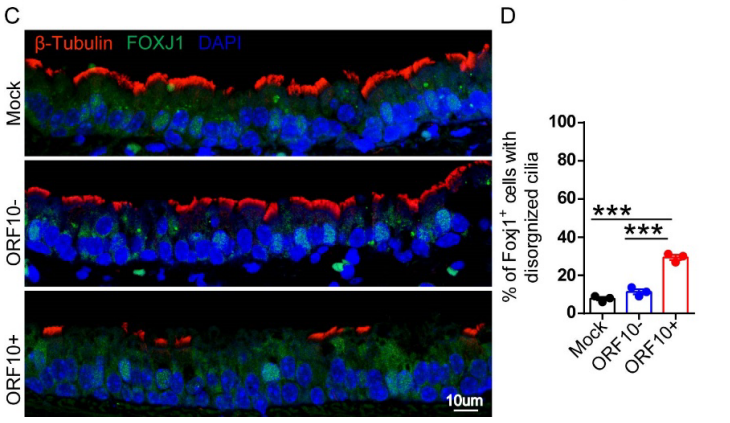
Response: Thanks for pointing out this problem. The dark pink lung symbols in rows 2 and 3 means virus infection, but not lung infiltration and congestion. We have revised this figure legend, as below:

"*(A) Schematic of the experimental design for the SARS-CoV-2 ORF10 infection system used in the present study. The dark pink lung symbols in rows 2 and 3 means virus infection.*"

- In panel 5B, it is not clear why the authors chose to use a basal cell marker to label bronchiolar tissue, rather than a ciliated cell marker that would have been more relevant to their study.

Response: Sorry for any confusion here and thanks for your suggestion. We now have evidence validating that ORF10 is expressed in ciliated cells based on the ciliated cell marker FOXJ1 (Fig. 7B). We stained epithelia for FOXJ1: we found an increased number of cells with disorganized cilia in the FOXJ1-positive cells in the Spike-lentivirus-ORF10 group compared with Spike-lentivirus and MOCK groups (Fig. R2C, D).

Figure R2



- The meaning of the histology images shown in Fig. 5D is unclear. It is difficult to see whether there is more congestion and lymphocytic infiltration in the ORF10+ than in the ORF10- condition. The explanations for the red arrows and dashed lines are missing.

Response: We now appreciate that the description of these results was flawed, and have removed this content from the revised manuscript. The red arrows indicate epithelial damage and the dashed lines indicate the boundary of the cilia layers.

Our revised manuscript presents this content as follows: (lines 381–383), (lines 1037–1038)

"*Further histological analysis of mice intranasally exposed to Spike-lentivirus-ORF10 revealed epithelial damage (Fig. 7D); these phenotypes are similar to observations of SARS-CoV-2 infected hACE2 mice (Fig. 8A).*"

"*The black arrows indicate epithelial damage and the dashed lines indicate the boundary of the cilia layers.*"

- As stated above, none of the in vivo data shown in Fig. 5 and 6 are quantified, and the authors provide no evaluation of the reproducibility of their findings.

Response: Thanks for your suggestion. We have repeated and quantified all of the *in vivo* experiments in Fig. 7 and Fig. 8. We found damaged cilia layers in the lung of mice exposed to Spike-lentivirus-ORF10 (Fig. 7D). Ciliated epithelial cells rate of 63.60% ± 1.806 was observed among the epithelial cells exposed to Spike-lentivirus-ORF10, compared to the 91.40% ± 1.030

and 91.80% ± 1.530 ciliated epithelial cells rate observed among the Spike-lentivirus group and MOCK group respectively (Fig. 7E, F). Immunofluorescence analysis of SARS-CoV-2 infection in hACE2 mice also displayed increased damaged cilia layers compared with the control group (Fig. 8A-C). These results showing that ORF10 can impair bronchiole cilia suggest that such damage may contribute to the pathogenesis of SARS-CoV-2 infection by facilitating the spread of SARS-CoV-2 deeper into the lung parenchyma.

- The authors provide remarkable proteomics data on ORF10-expressing 293-T cells, with a massive downregulation of proteins (352 downregulated and only 2 upregulated), involving primarily components and regulators of motile cilia. They mention in the discussion the study of Mena et al. (PNAS 2021), where a similar proteomic approach (TMT labeling followed by MS) in ORF10 expressing 293-T cells yielded very different results. Mena et al. reported fewer modulated proteins (8 downregulated and 91 upregulated), and no preferential loss of ciliary proteins. The authors should discuss the reasons for these discrepancies, as the downregulation of ciliary components is central to their message.

Response: Thank you for the very helpful comments and guidance to improve our study. To address the cause of the difference between our MS data and that of Mena *et al*.'s results (Mena et al., 2021), we carefully compared with the materials used in our and Mena *et al.*'s results. We found the ORF10 expression plasmid used in our study was different with that of Mena *et al.*'s results, and Mena *et al.*'s results did not include detected expression for ORF10. So we speculated that the protein level of ORF10 might be response for the difference between our and Mena *et al.*'s results. We first monitored the expression levels of ORF10 by transfecting HEK293T cells with pcDNA-ORF10-HA plasmids or pHAGE-ORF10-HA plasmids, the plasmids respectively used in our study and Mena *et al.*'s results. We found that ORF10-HA was successfully detected in pcDNA-ORF10-HA overexpressed samples, but not in pHAGE-ORF10-HA overexpressed samples (Fig. S2I), which was used in Mena *et al*.'s study. Then, we observed the protein level of

these cilium-related proteins after the transfection of pcDNA-ORF10-HA and pHAGE-ORF10-HA, and found these cilium-related proteins were dramatically decreased in pcDNA-ORF10-HA (Fig. S2L, M). These results suggest that the protein level of ORF10 is important for the stability of cilium-related proteins. To further confirm it, we have now measured the protein levels of these cilium-related proteins in a set of cell samples with a gradient of increasing ORF10 expression. We found that the protein level of these cilium-related proteins was dramatically decreased as ORF10 expression increased (Fig. S2J, K), indeed supporting that the protein level of ORF10 does affect the stability of these cilium-related proteins. Moreover, we reconstituted a Global Protein Stability (GPS) system with reporters to detect $CUL2^{ZYG11B}$ activity (*Nota Bene*: this is the experimental setup used in the Mena *et al.*'s study). We found that HEK293T cells transfected with pcDNA-ORF10-HA had a reduced GFP:DsRed ratio value compared to the empty vector control cells, which was similar with the effect of cells overexpressing ZYG11B (Fig. S2N). Therefore, the expression level of ORF10 may be account for the difference between our mass spectrometry results and those of Mena *et al*.'s study.

- It is not clear if the 293-T cells used for the proteomic analysis were transfected or transduced with a lentivector to induce ORF10 expression. Also, the authors mention in line 164 that the analysis was carried out across all replicates, but do not mention the number of replicates, nor the reproducibility of their findings when comparing different replicates. These points should be specified.

Response: We apologize that we didn't describe our data in a clearer way. The HEK293T cells used for the proteomic analysis were transfected with a pcDNA-ORF10-HA vector to induce ORF10 expression. We repeated three times for the proteomic analysis and confirmed the high reproducibility when comparing different replicates (Fig. S2A).

Our revised manuscript presents this content as follows: (lines 193–194)

"*Across three replicates for each group, reproducibility was high (Fig. S2A), and we obtained*

quantification data for a total of 8,820 proteins (Table S1)."

- The authors compared their set of proteins modulated by ORF10 to the set of proteins modulated by SARS-CoV-2 infection from the study of Bojkova et al. They observe a 22% overlap between the two datasets and mention in line 170 that most of the co-modulated proteins were reduced upon SARS-CoV-2 infection (Fig. S3B, C). This reduction is far from obvious visually (Fig. S3C), and the two datasets showed a trend for consistent variation at only one of the 4 time points tested (Fig. S3B). The overlap between the two datasets should be discussed in a more balanced fashion.

Response: Thanks for your suggestion. We have revised this content in the results.

Our revised manuscript presents this content as follows: (lines 196–202)

"A comparison of these proteins with data from a proteomics study of SARS-CoV-2 infection (Bojkova et al., 2020) showed ~22% (79/354) overlap between our proteomics data from ORF10-expressing cells and the SARS-CoV-2 infected proteome (Fig. S2B). Among these overlapped proteins, some proteins showed downregulated tendency in both ORF10 expression and SARS-CoV-2 infection (2h) (Fig. S2C, D), indicating the downregulation of some proteins after SARS-CoV-2 infection may be partially account for the ORF10."

- The statement on lines 218-220 that MG132 partially rescued ORF10-mediated promotion of protein degradation while 3-MA did not is questionable, as bands in lanes 3 and 4 of Fig. 4A look quite similar (except for ORF10 itself). The authors should repeat these experiments and show with statistics whether the differences between MG132 and 3-MA treatment are significant.

Response: Thanks for your suggestion. We have repeated and quantified the experiment. As shown in Fig. 4A-B, ORF10 could downregulate some cilium-related protein through ubiquitin-proteasome pathway excluding TTBK2.

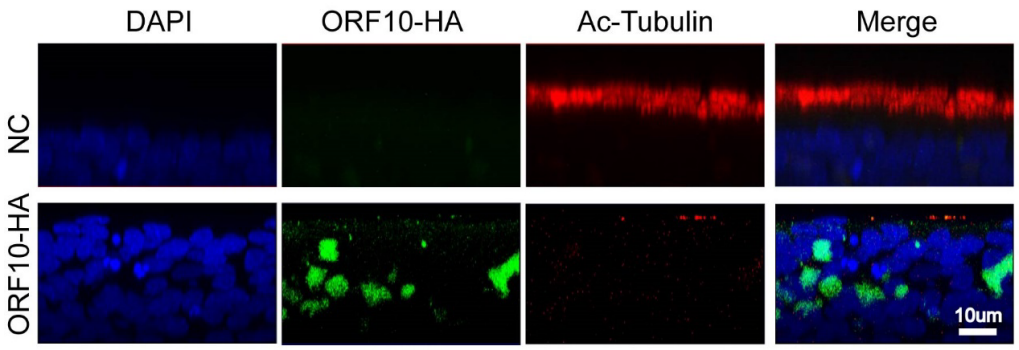
- The subcellular distribution of ORF10 is not precisely documented. In Figs. 3 and 4., what are the subcellular compartments involved? Also, could ORF10 be seen to colocalize with primary cilia before inducing their disappearance?

Response: Thanks for your questions. We found that ORF10 is primarily localized in the cytoplasm of NIH3T3 cells and MRC-5 cells, which is consistent with recently reported results (Zhang et al., 2020). As you suggested, in order to more accurately profile the localization of ORF10 in primary ciliated cells, we detected localization of ORF10 prior to the loss of cilia and found that ORF10 did not show any obvious co-localization with cilia (Fig. S3E, F). Similar results could also be found in ITF46 rescue experiments (Fig. 4K), in which ORF10 could not co-localize with restored cilia.

- If feasible, it would be informative to analyze ORF10 localization in primary multicilated cells, which are the actual target cells of SARS-CoV-2. Could the authors document the localization of ORF10 in multicilated cells at an early stage of infection, prior to the loss of cilia?

Response: Thanks for your suggestion. We have detected the localization of ORF10 in multiciliated primary human nasal epithelial cells (HNECs). Unfortunately, it is hard to observe the localization of ORF10 due to the several layers of cells in the multiciliated cell culture model (Fig. R4).

Figure R4



Minor points

- The affiliations are not numbered by order of appearance.

Response: Sorry for this mistake; we have corrected it.

- The reference format is not always consistent. Some of the references end with a + sign, like the first one.

Response: Sorry for this mistake; we have checked all references corrected them.

- Abbreviations are not always defined. For instance, Ac-Tubulin is to be defined in line 193.

Response: Sorry for this mistake; we have added it and checked other abbreviations throughout the manuscript.

- Explain that numbers below Western blot bands represent quantification of those bands.

Response: Sorry for confusion here. The number below western blot bands present the lane number; this has been explicated in the revised manuscript.

- Probable mistake in the legend of Fig. 4G (replace ORF10 by ZYG11B). Minus signs are

missing in the legend of Fig. 4E.

Response: Sorry for this mistake; we have corrected it.

- The manuscript would benefit from English editing. For instance, the word "for" is missing in line 141, and the word "cell" is missing in line 211.

Response: Sorry for this mistake; we have corrected it and seriously revised our manuscript.

We would again like to thank the Reviewer for the helpful guidance about how to improve our study.

Many thanks!

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April 18, 2022

RE: JCB Manuscript #202108015R

Prof. Wei Li
Chinese Academy of Sciences

Dear Prof. Li:

Thank you for submitting your revised manuscript entitled "SARS-CoV-2 ORF10 impairs cilia by enhancing CUL2 ZYG11B activity". The reviewers have now assessed your revised manuscript and their reviews are appended. We would be happy to publish your paper in JCB pending final revisions necessary to address the remaining reviewers' concerns and to meet our formatting guidelines (see details below).

While reviewers #2 and #3 are overall satisfied with revisions and support publication, reviewer #1 has a remaining concern on the proposed model that undermines the central conclusion of the paper -we agree with this reviewer that the data in Fig. 1J do not support your model of ORF10 simultaneously binding ZYG11B and E2D1. Thus, we would require that you carefully address reviewer #1's point, as well as the other remaining reviewers' points, before we could move forward with acceptance.

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully. Please go through all the formatting points paying special attention to those marked with asterisks.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles and Tools is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.

2) Figures limits: Articles and Tools may have up to 10 main text figures. Please note that main text figures should be provided as individual, editable files.

3) Figure formatting:
Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

*** Scale bars must be present on all microscopy images, including inset magnifications. Please include scale bars in inset magnifications of supplemental Figs 3A, 3C, 3E-G and 3J.

*** Also, please avoid pairing red and green for images and graphs to ensure legibility for color-blind readers. Please ensure that the particular red and green hues used in micrographs in main Figs 6B, 6D, 7B, 7E, 8B, and 9A are distinctive with any of the colorblind types. If not, please modify colors accordingly or provide separate images of the individual channels.

4) Statistical analysis:
*** Error bars on graphic representations of numerical data must be clearly described in the figure legend. Please describe error bars in main Figs 4D, 4H, 5F, 5H and supplemental Figs 2K, 4B, 4D, 4F.

*** The number of independent data points (n) represented in a graph must be indicated in the legend. Please indicate the number of independent data points in main Figs 4D, 4H, 5F, 5H and supplemental Figs 4B, 4D, 4F and specify whether it refers to technical replicates or independent samples/experiments.

Statistical methods should be explained in full in the materials and methods.

For figures presenting pooled data the statistical measure should be defined in the figure legends.

*** Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a

separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Please indicate in figure legends the statistical tests used in your experiments where appropriate.

*** As you used parametric tests in your study (i.e. t-tests), you should have first determined whether the data was normally distributed before selecting that test. In the stats section of the methods, please indicate how you tested for normality. If you did not test for normality, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title:
The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience.

The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described." Also, the materials and methods should be included with the main manuscript text and not in the supplementary materials.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods.

*** You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please include species for the FLAG, GST, HA and GFP antibodies purchased from Abmart.

8) Microscope image acquisition:
*** The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials:
There are strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental figures. There is no limit for supplemental tables.

*** Please note that supplemental figures and tables should be provided as individual, editable files.

A summary of all supplemental material should appear at the end of the Materials and Methods section (please see any recent JCB paper for an example of this summary).

11) eTOC summary:
*** A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person. It should begin with "First author name(s) et al..." to match our preferred style.

12) Conflict of interest statement:
JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests."

13) *** A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames and the CRediT nomenclature should be used (https://casrai.org/credit/).

14) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

15) Materials and data sharing: As a condition of publication, authors must make protocols and unique materials (including, but not limited to, cloned DNAs; antibodies; bacterial, animal, or plant cells; and viruses) described in our published articles freely available upon request by researchers, who may use them in their own laboratory only. All materials must be made available on request and without undue delay.

All datasets included in the manuscript must be available from the date of online publication, and the source code for all custom computational methods, apart from commercial software programs, must be made available either in a publicly available database or as supplemental materials hosted on the journal website. Numerous resources exist for data storage and sharing (see Data Deposition: https://rupress.org/jcb/pages/data-deposition), and you should choose the most appropriate venue based on your data type and/or community standard. If no appropriate specific database exists, please deposit your data to an appropriate publicly available database.

16) Animal studies statement:
All animal and human studies must be conducted in compliance with relevant local guidelines, such as the US Department of Health and Human Services Guide for the Care and Use of Laboratory Animals or MRC guidelines, and must be approved by the authors' Institutional Review Board(s). A statement to this effect with the name of the approving IRB(s) must be included in the Materials and methods section.

17) Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the files will be directly linked to specific figures in the published article.

As your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

**It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images

upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.**

The license to publish form must be signed before your manuscript can be sent to production. A link to the electronic license to publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements before choosing the appropriate license.

Additionally, JCB encourages authors to submit a short video summary of their work. These videos are intended to convey the main messages of the study to a non-specialist, scientific audience. Think of them as an extended version of your abstract, or a short poster presentation. We encourage first authors to present the results to increase their visibility. The videos will be shared on social media to promote your work. For more detailed guidelines and tips on preparing your video, please visit https://rupress.org/jcb/pages/submission-guidelines#videoSummaries.

Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days. Please let us know if any complication preventing you from meeting this deadline arises and we can work with you to determine a suitable revision period.

Please contact the journal office with any questions, cellbio@rockefeller.edu.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Maxence Nachury
Monitoring Editor
Journal of Cell Biology

Lucia Morgado-Palacin, PhD
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In this revised version the authors have addressed many of the points raised in the previous issue. Although most of them are now satisfactory, there is a major remaining issue in the binding experiments, where the authors claim simultaneous binding, whereas they clearly show competitive binding of E2D1 and ZYGB1 for ORF10; This really is a major problem, as it seems to me that competitive binding in fact excludes the proposed model and cannot be glossed over. The fact that you can precipitate simultaneously from bulk just means that under those conditions not both types of binding are saturated, but not that these bind simultaneously. This manuscript tries to address very many issues, but if that is the case they do need to be individually correct. With these data the major conclusion that simultaneous binding of these three factors activates ubiquitination is shown to be incorrect and therefore this manuscript is not publishable.

There were additional issues noted while reading, that could be helpful for the authors for future reference, e.g. if they decide to split the IFT46 part from the problematic first half.

- There are labelling issues on fig 1I-K in the main text;

- page 10 line 204:sentence unclear: the downregulation of some proteins after SARS-CoV-2 infection may be partially account for the ORF10.

- Fig S2G, S2H; it is not clear what is meant by Elledge et al, there is no such paper, should be mena et al; use the surface area of the venn diagram to show the level of overlap; the lower expression level in Mena et al could be much more physiologically relevant, need to compare to infection i guess, but that may be hard;

- For future reference: comparison of the delineation of the C2 domain in IFT46 shows that domain borders can be refined; currently 2 separate folded regions are affected by c2 deletion

- The reviewer comments on lenalidomide etc were misunderstood: they were meant as an alternative example of an E3 ligase

being redirected to new targets. Either add this discussion (which is not absolutely needed, but would zoom out on the issue of redirection of E3 ligases) or remove the reference to these compounds

- The materials and methods of the SPR analysis is missing. It is good to show the raw curves, but it is aso necessary to show the fitting of the data in 1I; What is the fitting model? hard to imagine a simple 1:1 fit would work, with those off-rate kinetics.Alternatively do equibrium fitting but explain the issues and choices made (in materials and methods).

Reviewer #2 (Comments to the Authors (Required)):

The revisions have addressed my concerns.

Reviewer #3 (Comments to the Authors (Required)):

Wang et al. are providing a substantially revised manuscript documenting the role of ORF10 in increasing the activity of the CUL2-ZYG11B ubiquitin ligase complex and in inducing the loss of motile cilia. In particular, they are giving a plausible explanation as to why their findings differ from those of Meena et al., by showing that they used a more efficient ORF10 expression vector in the present study, and that ORF10 effects are dose dependent.

The authors have also addressed this reviewer's concerns regarding the mouse model experiments, which are now better described and quantified. Importantly, they are also providing additional data substantiating their claim in a reconstructed human nasal epithelium model, by showing that ORF10 transduction does lead to a clear loss of motile cilia in this primary cell system.

The authors have also strengthned the biochemical part of the manuscript, by mapping the interaction between ORF10 and the ciliary transport protein IFT46, and by measuring the affinity of the ORF10/ZYG11B and ORF10/UBE2D1 interactions by SPR. They are also providing data to document the reproducibility of the proteomic analysis.

Overall, the authors have addressed all of this reviewer's concerns, and have significantly improved the manuscript. Their findings are important to the understanding of SARS-CoV-2 pathogenesis, as they help explain the anosmia/ageusia frequently associated to COVID-19.

Minor points:

- The competition SPR experiments that are supposed to be inserted in Fig. 1 are not shown, and the descriptions of panels H to K are mixed up in the figure legend.

- The authors are providing additional immunofluorescence experiments to determine the subcellular localization of ORF10, which turns out to be cytoplasmic. This finding suggests an indirect mode of action for ORF10, which does not localize to cilia, but nevertheless impacts the stability of ciliary proteins, as convincingly shown by the authors. These IF experiments, and those showing the increase of FOXJ1+ cells with disorganized cilia, could be added to the manuscript, rather than be shown exclusively to the reviewers.

Point by point responses:

We appreciate the careful reviewing from Dr. Nachury, Dr. Morgado-Palacin and the three experts for their time and professional opinions. We considered and reviewed their comments carefully, and present our point-to-point response at the following paragraph regarding these comments.

Editor：

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised.

Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.

1) Text limits: Character count for Articles and Tools is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.

Response: We have carefully revised the manuscript and the character count in this manuscript is 28,043.

2) Figures limits: Articles and Tools may have up to 10 main text figures. Please note that main text figures should be provided as individual, editable files.

Response: We have 9 main text figures, and have provided individual and editable figures.

3) Figure formatting:

Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

Response: We have labeled molecular weight or nucleic acid size markers on all gel electrophoresis.

*** Scale bars must be present on all microscopy images, including inset magnifications. Please include scale bars in inset magnifications of supplemental Figs 3A, 3C, 3E-G and 3J.

Response: We have added scale bars in inset magnifications of supplemental Figs 3A, 3C, 3E-G and 3J.

*** Also, please avoid pairing red and green for images and graphs to ensure legibility for color-blind readers. Please ensure that the particular red and green hues used in micrographs in main Figs 6B, 6D, 7B, 7E, 8B, and 9A are distinctive with any of the colorblind types. If not, please modify colors accordingly or provide separate images of the individual channels.

Response: We have modified colors in Figs 6B, 6D, 7B, 7E, 8B, 9A and supplemental Figs 2M.

4) Statistical analysis:

*** Error bars on graphic representations of numerical data must be clearly described. Please describe error bars in main Figs 4D, 4H, 5F, 5H and supplemental Figs 2K, 4B, 4D, 4F.

Response: We have described error bars in the figure legend of the main Figs 4D, 4H, 5F, 5H and supplemental Figs 2K, 4B, 4D, 4F.

*** The number of independent data points (n) represented in a graph must be indicated in the legend. Please indicate the number of independent data points in main Figs 4D, 4H, 5F, 5H and supplemental Figs 4B, 4D, 4F and specify whether it refers to technical replicates or independent samples/experiments.

Response: We have indicated the number of independent data points in the figure legends.

Statistical methods should be explained in full in the materials and methods.

Response: We have explained the statistical methods in the materials and methods.

"Statistical analysis

All experiments were repeated at least three times and all data were expressed as mean ± SEM. SPSS software (IBM SPSS statistics 26) was used for testing normality of the data. The statistical significance of the differences between the mean values for the different genotypes was measured by two-tailed unpaired student's t test. Statistical analysis and figures were made with GraphPad Prism 9. The data were considered significant when the P value was less than 0.05 (*), 0.01 (**), or 0.001 (***)."

For figures presenting pooled data the statistical measure should be defined in the figure legends.

Response: Pooled data were not included in this study.

*** Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Please indicate in figure legends the statistical tests used in your experiments where appropriate.

Response: We have indicated the statistical tests and the parameters of the test in figure legends and "Statistical analysis" section.

*** As you used parametric tests in your study (i.e. t-tests), you should have first determined whether the data was normally distributed before selecting that test. In the stats section of the methods, please indicate how you tested for normality. If you did

not test for normality, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

Response: We have tested the data for normality by using SPSS software (IBM SPSS statistics 26) before selecting t-tests. The descriptions of the "Statistical analysis" have been revised as below:

"Statistical analysis

All experiments were repeated at least three times and all data were expressed as mean ± SEM. SPSS software (IBM SPSS statistics 26) was used for testing normality of the data. The statistical significance of the differences between the mean values for the different genotypes was measured by two-tailed unpaired student's t test. Statistical analysis and figures were made with GraphPad Prism 9. The data were considered significant when the P value was less than 0.05 (*), 0.01 (**), or 0.001 (***)."

5) Abstract and title:

The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience.

Response: We have revised the abstract.

The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

Response: The title has 63 characters including spaces.

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described." Also, the materials and methods should be included with the main manuscript text and not in the supplementary materials.

Response: We have provided full descriptions in Materials and methods section.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods.

Response: We have provided the sequences of RNAi in the materials and methods.

*** You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please include species for the FLAG, GST, HA and GFP antibodies purchased from Abmart.

Response: We have added the species of antibodies in the methods.

"Mouse antibody to FLAG (1:2000, M20008L), mouse antibody to GST (1:2000, M20007L), mouse antibody to HA (1:1000, M20003L), mouse antibody to GFP (1:1000, M20004L) and mouse antibody to MYC (1:1000, M20002M) antibodies were purchased from Abmart (Shanghai, China)."

8) Microscope image acquisition:

*** The following information must be provided about the acquisition and processing of images:

a. Make and model of microscope

b. Type, magnification, and numerical aperture of the objective lenses

c. Temperature

d. imaging medium

e. Fluorochromes

f. Camera make and model

g. Acquisition software

h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

Response: We have provided detail information about the acquisition and processing of images in the methods.

"Images were collected immediately using a confocal microscope (LSM 880 Meta plus Zeiss Axiovert Zoom; Zeiss) equipped with a 63×(NA=1.40) oil-immersion objective lens (Plan-Apochromat; Zeiss) and acquired by highly sensitive PMT at RT. Images were analyzed by using ZEN software (2011_Lite_x64) and aligned by Adobe Illustrator (CS4)."

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

Response: References have cited by ***Journal of Cell Biology*** style.

10) Supplemental materials:

There are strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental figures. There is no limit for supplemental tables.

Response: We have 5 supplemental figures in our study.

*** Please note that supplemental figures and tables should be provided as individual, editable files.

Response: We have provided individual and editable supplemental figures.

A summary of all supplemental material should appear at the end of the Materials and Methods section (please see any recent JCB paper for an example of this summary).

Response: We have provided the summary of all supplemental material at the end of the Materials and Methods section.

11) eTOC summary:

*** A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person. It should begin with "First author name(s) et al..." to match our preferred style.

Response: We have added the summary on the title page.

"*Wang et al. report that the SARS-CoV-2 ORF10 promotes IFT46 degradation via stimulating CUL2^{ZYG11B} activity, thereby impairing both cilia biogenesis and maintenance. The study provides a pathological mechanism connecting COVID-19 symptoms with cilia dysfunction.*"

12) Conflict of interest statement:

JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests."

Response: We have added the statement:

"*The authors declare no competing financial interests.*"

13) *** A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames and the CRediT nomenclature should be used (https://casrai.org/credit/).

Response: We have revised the author contribution section according with your suggestion.

"Liying Wang, Chao Liu, Bo Yang, Haotian Zhang and Jian Jiao: conceptualization, investigation, data curation, validation, project administration, methodology, formal analysis, and writing-original draft. Ruidan Zhang, Sai Xiao and Yinghong Chen: investigation and methodology. Shujun Liu and Yueshuai Guo: investigation, methodology and formal analysis. Bo Liu and Yanjie Ma: methodology. Xuefeng Duan: resources. Mengmeng Guo and Bingbing Wu: investigation. Xiangdong Wang and

Yaoting Gui: resources. Xingxu Huang and Haitao Yang: supervision. Min Fang: resources and supervision. Luo Zhang and Shuguang Duo: conceptualization, resources and supervision. Xuejiang Guo: conceptualization, data curation, methodology, resources and supervision. Wei Li: conceptualization, resources, supervision, funding acquisition, writing—original draft, review, and editing."

14) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

Response: We have provided the ORCID IDs for the contributing authors.

"Liying Wang: http://orcid.org/0000-0002-3093-1311, Chao Liu: http://orcid.org/0000-0002-8844-0697, Bo Yang: http://orcid.org/0000-0003-1486-2341, Haotian Zhang: http://orcid.org/0000-0002-7181-0962, Jian Jiao: http://orcid.org/0000-0003-0716-2654, Ruidan Zhang: http://orcid.org/0000-0002-4924-8766, Shujun Liu: http://orcid.org/0000-0003-4066-4891, Sai Xiao: http://orcid.org/0000-0002-9551-2249, Yinghong Chen: http://orcid.org/0000-0003-2208-4105, Bo Liu: http://orcid.org/0000-0002-9273-054X, Yanjie Ma: http://orcid.org/0000-0002-9000-1108, Xuefeng Duan: http://orcid.org/0000-0002-5281-1425, Yueshuai Guo: http://orcid.org/0000-0002-2719-1244, Mengmeng Guo: http://orcid.org/0000-0002-0979-3487, Bingbing Wu: http://orcid.org/0000-0001-5890-4377, Xiangdong Wang: http://orcid.org/0000-0002-0409-322X, Xingxu Huang: http://orcid.org/0000-0001-8934-1247, Haitao Yang: http://orcid.org/0000-0002-1875-3268, Yaoting Gui:

https://orcid.org/0000-0002-5299-8330, Min Fang: http://orcid.org/0000-0002-5278-1430, Luo Zhang: http://orcid.org/0000-0002-0910-9884, Shuguang Duo: http://orcid.org/0000-0003-4236-3697, Xuejiang Guo: http://orcid.org/0000-0002-0475-5705, Wei Li: http://orcid.org/0000-0002-6235-0749."

15) Materials and data sharing: As a condition of publication, authors must make protocols and unique materials (including, but not limited to, cloned DNAs; antibodies; bacterial, animal, or plant cells; and viruses) described in our published articles freely available upon request by researchers, who may use them in their own laboratory only. All materials must be made available on request and without undue delay.

Response: We added the statement in Data availability section:

"*The authors declare that all other data and materials supporting the findings of this study are available within the article and should be addressed to the corresponding author.*"

All datasets included in the manuscript must be available from the date of online publication, and the source code for all custom computational methods, apart from commercial software programs, must be made available either in a publicly available database or as supplemental materials hosted on the journal website. Numerous resources exist for data storage and sharing (see Data Deposition: https://rupress.org/jcb/pages/data-deposition), and you should choose the most appropriate venue based on your data type and/or community standard. If no appropriate specific database exists, please deposit your data to an appropriate publicly available database.

Response: We have deposited our mass spectrometry proteomics data to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) with the dataset identifier PXD031842 and provided it in our revised manuscript.

16) Animal studies statement:

All animal and human studies must be conducted in compliance with relevant local guidelines, such as the US Department of Health and Human Services Guide for the Care and Use of Laboratory Animals or MRC guidelines, and must be approved by the authors' Institutional Review Board(s). A statement to this effect with the name of the approving IRB(s) must be included in the Materials and methods section.

Response: We have added the statement the Materials and methods section.

"All of the animal experiments were performed according to approved institutional animal care and use committee (IACUC) protocols (#2021-002) of the Institute of Zoology, Chinese Academy of Sciences."

"This study was approved by the Ethics Committee of Beijing Tongren Hospital, and all subjects signed informed consent before recruitment in the study."

17) Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the files will be directly linked to specific figures in the published article.

As your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated

main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

Response: We have uploaded all Source Data Figures according with your suggestion.

Reviewer #1 (Comments to the Authors (Required)):

In this revised version the authors have addressed many of the points raised in the previous issue. Although most of them are now satisfactory, there is a major remaining issue in the binding experiments, where the authors claim simultaneous binding, whereas they clearly show competitive binding of E2D1 and ZYGB1 for ORF10; This really is a major problem, as it seems to me that competitive binding in fact excludes the proposed model and cannot be glossed over. The fact that you can precipitate simultaneously from bulk just means that under those conditions not both types of binding are saturated, but not that these bind simultaneously. This manuscript tries to address very many issues, but if that is the case they do need to be individually correct. With these data the major conclusion that simultaneous binding of these three factors activates ubiquitination is shown to be incorrect and therefore this manuscript is not publishable.

Response: We would like to thank Reviewer 1 for the thorough and highly insightful review and for the helpful comments. After rethinking of the results in Figure 1J, I totally agree with that our data could not support the simultaneous binding of

ZYG11B-ORF10-UBE2D1, as once binding with ZYG11B, the ORF10 cannot interact with UBE2D1. Therefore, the previous model cannot explain why ORF10 could enhance the E3 ligase activity of $CUL2^{ZYG11B}$. Thus, we have removed this part in the revised manuscript. And the removing of these results does not affect the conclusion that ORF10 could directly interact with ZYG11B to promote the E3 ligase activity of $CUL2^{ZYG11B}$.

There were additional issues noted while reading, that could be helpful for the authors for future reference, e.g. if they decide to split the IFT46 part from the problematic first half.

Response: We thank Reviewer 1 for his/her kindly help in improving the manuscript. We have removed the incomplete model of ORF10 simultaneously binding ZYG11B and E2D1 and focus on ORF10 impairs cilia via promoting IFT46 degradation.

- There are labelling issues on fig 1I-K in the main text;

Response: Thanks for your helpful guidance here! We have modified it.

- page 10 line 204: sentence unclear: the downregulation of some proteins after SARS-CoV-2 infection may be partially account for the ORF10.

Response: We have corrected it.

"*The downregulation of some proteins after SARS-CoV-2 infection may be partially account for the expression of ORF10*".

- Fig S2G, S2H; it is not clear what is meant by Elledge et al, there is no such paper, should be mena et al; use the surface area of the venn diagram to show the level of overlap; the lower expression level in Mena et al could be much more physiologically relevant, need to compare to infection i guess, but that may be hard;

Response: Sorry for this mistake, we have corrected it.

- For future reference: comparison of the delineation of the C2 domain in IFT46 shows that domain borders can be refined; currently 2 separate folded regions are affected by c2 deletion

Response: Thanks for your suggestion. We would like to refine the borders of IFT46 C2 domain in the future work.

- The reviewer comments on lenalidomide etc were misunderstood: they were meant as an alternative example of an E3 ligase being redirected to new targets. Either add this discussion (which is not absolutely needed, but would zoom out on the issue of redirection of E3 ligases) or remove the reference to these compounds.

Response: Thanks for your suggestion. We now understand ORF10 could stimulate the E3 ligase activity of $CUL2^{ZYG11B}$ to promoter the degradation of IFT46, and the functional redirection is similar to that of these compounds, but it is not absolutely needed in our discussion. Thus, we have removed the reference to these compounds in discussion as your suggestion.

- The materials and methods of the SPR analysis is missing. It is good to show the raw curves, but it is also necessary to show the fitting of the data in 1I; What is the fitting model? hard to imagine a simple 1:1 fit would work, with those off-rate kinetics. Alternatively do equibrium fitting but explain the issues and choices made (in materials and methods).

Response: Sorry for this problem. We have added the fitting curve in Fig. 2B and the binding kinetic analysis in the methods section.

"*Biolayer interferometry*

The binding kinetic and dissociation constant of ZYG11B-ORF10 were performed using an Octet Red 96 (Forté Bio, Fremont, CA) in assay buffer (PBS, 0.002% (v/v) Tween 20). ZYG11B were immobilized to a level of 0.5 nM on streptavidin-coated biosensors (Forté Bio, Fremont, CA). To verify that no nonspecific binding was present during the assay, non-functionalized biosensors were used as a control by

measuring in parallel all ligand concentrations as well as running buffer. For ORF10 a two-fold dilution series from 6.25 nM–0.39 nM was employed. Data analysis was performed using Data Analysis software 9.0.0.14 (Forté Bio, Fremont, CA) using the 2:1 heterogenous ligand-binding model. The binding curves were exported to excel for plotting of curves."

Reviewer #2 (Comments to the Authors (Required)):

The revisions have addressed my concerns.

Response: Thank you very much!

Reviewer #3 (Comments to the Authors (Required)):

Wang et al. are providing a substantially revised manuscript documenting the role of ORF10 in increasing the activity of the CUL2-ZYG11B ubiquitin ligase complex and in inducing the loss of motile cilia. In particular, they are giving a plausible explanation as to why their findings differ from those of Meena et al., by showing that they used a more efficient ORF10 expression vector in the present study, and that ORF10 effects are dose dependent.

The authors have also addressed this reviewer's concerns regarding the mouse model experiments, which are now better described and quantified. Importantly, they are also providing additional data substantiating their claim in a reconstructed human nasal epithelium model, by showing that ORF10 transduction does lead to a clear loss of motile cilia in this primary cell system.

The authors have also strengthned the biochemical part of the manuscript, by mapping the interaction between ORF10 and the ciliary transport protein IFT46, and by measuring the affinity of the ORF10/ZYG11B and ORF10/UBE2D1 interactions by

SPR. They are also providing data to document the reproducibility of the proteomic analysis.

Overall, the authors have addressed all of this reviewer's concerns, and have significantly improved the manuscript. Their findings are important to the understanding of SARS-CoV-2 pathogenesis, as they help explain the anosmia/ageusia frequently associated to COVID-19.

Minor points:

- The competition SPR experiments that are supposed to be inserted in Fig. 1 are not shown, and the descriptions of panels H to K are mixed up in the figure legend.

Response: Thank you for the very helpful comments and guidance to improve our study.

As reviewer 1 suggestion, we have removed this part in the revised manuscript. The descriptions of the figure legend have been revised as below:

"To detect the binding affinity, we performed Bio-Layer Interferometry (BLI) experiment and found that ORF10-ZYG11B had a pretty high binding affinity (Fig. 1B)."

- The authors are providing additional immunofluorescence experiments to determine the subcellular localization of ORF10, which turns out to be cytoplasmic. This finding suggests an indirect mode of action for ORF10, which does not localize to cilia, but nevertheless impacts the stability of ciliary proteins, as convincingly shown by the authors. These IF experiments, and those showing the increase of FOXJ1+ cells with disorganized cilia, could be added to the manuscript, rather than be shown exclusively to the reviewers.

Response: Thanks for your suggestion. We have added these results in Fig. S5.