

Peer Review File

Manuscript Title: Papain-like protease regulates SARS-CoV-2 viral spread and innate immunity

Editorial Notes:

Redactions – Third Party Material

Parts of this Peer Review File have been redacted as indicated to remove third-party material.

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referee #1 (Remarks to the Author):

Shin et al. describe a broad biochemical and structural study on the papain-like protease (PLpro) of the novel coronavirus, SARS-CoV-2. First, they compared the deubiquitinating, deISGylating, and deneddylating activities of the enzyme with those of SARS-CoV PLpro. Surprisingly, it turned out that SARS-CoV-2 strongly prefers removal of ISG15 over removal of ubiquitin (Ub) and di-Ub, whereas the reverse is true for SARS-CoV PLpro. Next, the authors determined the crystal structure of the complex between SARS-CoV-2 PLpro and murine ISG15. They found that in this complex, the two Ub-like domains of ISG15 have a mutual orientation that is different from the one seen in human ISG15 on its own. Also, there were some differences between the details of interactions between PLpro and di-Ub in case of SARS-CoV and PLpro and ISG15 in case of SARS-CoV-2, but it is not obvious in which way they would determine the differences in specificity of the two enzymes.

To see whether the cause of the specificity differences lies perhaps in the dynamics of the interactions, the authors performed molecular dynamics simulations. However, in my view, these did not reveal additional findings concerning the differences in specificity.

The authors went on to test the compound GRL0617, which is a proven inhibitor for the SARS-CoV PLpro, and found that it also efficiently inhibits SARS-CoV-2 PLpro.

Next, Shin et al. investigated the interactomes of SARS-CoV-2 PLpro and SARS-CoV PLpro. This revealed that the enzyme of the novel coronavirus mainly affects signaling proteins involved in the control of interferon production, whereas SARS-CoV PLpro interfered with proteins of the NFκB pathway that mainly controls cytokine production.

This manuscript presents very interesting data. Unfortunately, the differences between the substrate specificities of these highly similar proteins are not readily explained by comparing their three-dimensional structures. Yet, the findings are of very high importance and relevance.

Individual concerns:

(1) It is not clear whether the authors work with the full-length SARS-CoV-2 PLpro or cut off the N-terminal ubiquitin-like domain (Ubl), as it is often done when the full-length enzyme refuses to crystallize.

(2) line 50: "mortality" should be replaced by "case - fatality rate". "Mortality" is the fraction of the total population succumbing to the viral infection and is extremely low. "Case - fatality rate" is the fraction of diseased people who die from the infection.

(3) line 52: The MERS outbreak of 2012 cannot be called "global". The virus only surfaced on the Arab peninsula and, in 2015, there was a travel-related outbreak in South Korea. It should also be mentioned that the MERS-CoV outbreak on the Arab peninsula is still ongoing.

(4) line 55: "a critical step" or what? Something missing here.

(5) line 95: reference missing for MERS-CoV PLpro in complex with human ISG15

(6) line 97: reference missing for crystal structure of ISG15 on its own.

(7) line 99: reference missing for SARS-CoV PLpro in complex with C-terminal domain of ISG15

(do not provide only PDB code)

(8) line 181: reference missing

(9) lines 182, 189: IkappaB

(10) line 190: NF-kappaB

(11) lines 317, 318, 404, 615, 618, 619, 624: references for crystal structures missing (do not only provide PDB codes!).

(12) line 354: Equal amounts ...

(13) lines 381, 382, 386: ... as previously described

(14) line 383: purified by size-exclusion chromatography

(15) line 386: CSN should be explained upon first occurrence.

(16) line 404, 415, 424: What is the apo form of the PLpro? An apoenzyme is an enzyme that lacks an essential cofactor. In case of the PLpro, the apoenzyme would be the protein lacking the zinc ion bound to the fingers domain. With all components present, the enzyme would be called a holoenzyme. However, this is not what the authors mean. Unfortunately, the term "apo" is being widely misused by structural biologists to indicate the absence of the substrate of an enzyme. (What would be the holoenzyme then?). It is the obligation of authors and editors to keep the language of science as clean and unambiguous as possible and therefore, the incorrect usage of the term "apo" should be banned.

(17) line 418: Is this the BL2 loop?

(19) line 428: I am not sure about the catalytic His being protonated. This would assume that the active-site Cys is negatively charged. This is true for papain but has, to the best of my knowledge, not been demonstrated for coronavirus PLpros.

(20) line 624: Why is the PLpro called PLP2 here?

(21) Reference 11: Something seems to have gone wrong here.

(22) The table with crystallographic data is not readable, in parts.

Referee #2 (Remarks to the Author):

In "Inhibition of papain-like protease PLpro blocks SARS-CoV-2 spread and promotes viral entry", Shin et al. characterize the host-directed effects of PLpro from SARS-CoV-2. Strengths of this study are the frequent and thorough comparisons of PLpro from SARS-CoV and SARS-CoV-2, thorough structural and biochemical characterization of PLpro, and the use of functional assays to demonstrate the relevance of their proposed mechanism during infection in cells. The major concern is novelty. The crystal structure of a CoV PLP in complex with ISG15 has been reported (PMID: 28438633), and GRL0617 is well characterized as inhibiting CoV replication by targeting PLP (PMID: 18852458). Thus, the major findings of this study are largely expected based on the high similarities between SARS-CoV-2 and SARS-CoV. The authors do reports some notable distinctions between the two PLPs which are very interesting and add new insight into the distinct biology of these viruses. If the authors demonstrated antiviral activity of GRL0617 against SARS-CoV-2 in more relevant systems, this would likely significantly improve the broad interest in this

study. Additionally, some of the main conclusions would benefit from additional supporting evidence, as outlined below.

Major comments

1. Related to figure 2C: The authors suggest based on structural data and modelling that PLproSARS L76 interacts with ubiquitin I44, whereas PLproCoV2 T75 does, perhaps explaining the differences in substrate specificity. It should be easy to introduce reciprocal mutations into the two proteases and test this prediction functionally, especially since the authors test LOF mutations for the CoV-2 protease in 2D. Functional data would greatly enhance this conclusion.
2. The authors observe that PLproSARS associates with SERPINs while PLproCoV-2 does not and note that SERPIN family proteins are known to inhibit papain-like protease activity. This could be another compelling difference between PLproSARS and PLproCoV-2 if the latter has somehow adapted to avoid potential inhibition by SERPINs. Functional assays could address this and potentially enhance the novelty of this study.
3. Figure 5d: The authors quantify viral RNA in supernatant as a readout for viral production. The gold standard assays for virus production are those that discriminate between defective particles and truly infectious particles, such as plaque assays and TCID₅₀ assays. Such an assay would complement the existing data.
4. Figure 5e: It appears that GRL-0671 reduces the detection of free ISG15 in the IP in the absence of SARS-CoV-2 infection. Could the authors comment on this? Is it possible that GRL-0671 is impacting either loose association of ISG15 with IRF3 or ISG15 levels at basal states? Probing the input membrane for ISG15 would be helpful, as would an isotype control to demonstrate the specificity of the IP.
5. It is unclear why the authors invoke discussion of the DNA sensing pathway in Lines 169-172. CoV are RNA viruses. They would not activate innate immunity through cGAS-STING-IRF3, but rather through MAVS-IRF3. And the Plos One paper (ref 33) claiming PLP antagonism of STING did not confirm this with live virus. It should thus be taken with a grain of salt. This section should be edited to reflect more accurately the known CoV sensing pathways.

Minor points & typographical errors

line 55 – “a critical xx for” (missing word)

line 181 – “[ref]” missing

line 185 – Viral “entry” is not mimicked by poly(I:C) treatment – sensing of viral nucleic acid is mimicked by this assay.

Figure 4b is not explicitly discussed in the text.

Line 232 – the study does not necessarily establish that PLpro is “an equally promising target” as 3CLpro, as no comparisons are made within this text. It would be more appropriate to simply state that PLpro is “an additional promising target” or something along those lines.

Referee #3 (Remarks to the Author):

This manuscript by Shin and co-workers describes an impressive array of biochemical assays, structural studies, and pharmacological studies that document the multifunctional role of the papain-like protease (PLpro) during SARS-CoV-2 replication. These studies leveraged 15 years of previous work, but instead of acknowledging the previous work in the background section of the paper, the authors instead selectively incorporate the previous studies into the presentation of their own experiments, which implies that the previous studies support their results, instead of the other way around. The authors should re-write this paper in a way that conveys to other researchers around the world how previous studies that documented the multifunctional nature of all coronavirus papain-like proteases, structural studies that identified residues that interact with ubiquitin, screening of libraries to identify non-covalent inhibitors, and co-crystal structural studies that identified the site bound the inhibitor, can all be leveraged to make rapid progress toward developing therapeutics that inhibit the replication of SARS-CoV-2, and have potential for treating

COVID-19 patients.

Comments for the authors' consideration:

1. Abstract: CoV proteases process the viral polyprotein to generate a functional replicase complex. It is not clear what is meant by viral maturation, so this should be deleted.
2. Abbreviations: The typical presentation is to name the virus first, and then the enzyme, so SC-PLpro and SC2-PLpro would be more appropriate than the current superscript designation.
3. Abstract: Would it be accurate to state that it was by comparing previous structural information on binding of GRL-0617 into a flexible loop adjacent to the PLpro active site, to the new structural information on SC2-PLpro which documents the same flexible loop adjacent to the active site, that suggested that the previously identified inhibitor would block the active site of both enzymes? It is not clear why you would need to leverage the SC2-PLpro-ISG15 structure to appreciate the similarities of the two enzymes and the presence of the flexible loop.
4. Background section: Comparing the recent progress on 3CLpro to PLpro is not really the important issue here. Instead focus on how leveraging previous studies of PLpro allows researchers to make rapid progress on identifying inhibitors of SARS-CoV-2 PLpro.
5. Line 56: remove maturation, release and further spread, as this is not the function of these proteases.
6. Line 59-62, reference the studies that document CoV PLpro multifunctionality and inhibition of the innate immune signaling, not reviews on general innate immunity (ref 9-12).
7. Line 70 and Figure 1. The teeter-totter diagram make it look like it is all or none, but the results indicate the multifunctional nature of both enzymes, with this expressed region of SC2-PLpro exhibiting more robust de-ISGylating activity. The authors should note that PLpro is part of the larger nonstructural protein 3 (nsp3). The context of PLpro within nsp3 may further influence enzyme activity.
8. Line 125: Provide more information on how GRL-0617 was identified by high through-put screening and SAR modification to help the readers understand how this antiviral was identified.
9. Line 132: Reference the lack of activity of this type of inhibitor for MERS-PLpro (Kilianski et al., 2014), as this is already known.
10. Lines 145-147: These results confirm and extend the previous studies that documented that GRL-0617 is an effective inhibitor for SARS-CoV, suggesting that it may be more broadly effective as an inhibitor to the many SARS-related CoVs that are known to exist in bat populations around the world.
11. Line 185-193 and Figs 4e and 4f. These overexpression assays and stimulation by poly(I:C) and TNF-alpha may not reflect anything about the biological function of PLpro in the context of virus replication, so should be deleted. During virus replication, PLpro is part of a membrane-associated complex, not free in the cell, so the activity reflected in this assay may or may not reflect the activity of the enzyme in the context of the membrane-associated viral replication complex.
12. Line 205: remove maturation and release. Inhibiting the protease stops the assembly of the viral replication complex, which is necessary for on-going viral RNA synthesis. If there is no RNA synthesis, then the production of infectious progeny virus stops.
13. Fig 5e. Excellent experiment and clear result. It would be even better if you included infection with SARS-CoV for a direct comparison with SARS-CoV-2. You may see a very similar result. This experiment would help to determine if the differences you see in substrate preferences in the overexpression studies are also reflected in the activity of the enzyme when it is part of the replication complex.
14. Line 217: blocking processing and assembly of the replication complex and viral RNA synthesis (not virus release)
15. Lines 219-228. This is speculation that PLpro is a differential virulence factor among CoVs, and Niemeyer et al., 2018 PLoS Pathogens should be cited here. It should also be noted that there may be many virulence factors that contribute to the high transmissibility of SARS-CoV-2 and distinct pathologic outcomes (SARS-CoV-2 has a lower case fatality rate than SARS-CoV, which was 10%). Multiple factors contribute to the ability of SARS-CoV-2 to cause a pandemic that differs in transmissibility (higher than SARS-CoV) and morbidity (lower than SARS-CoV).
16. Fig 5h. MDA-5 is the PRR for CoVs, not RIG-I; also the right hand side should point to the

double membrane vesicles where nsp3 resides, not to virus particles.

17. Line 229-234 – It was already established for SARS-CoV that PLpro is a target for therapeutics. Delete the comparison of 3CLpro (Mpro) and PLpro, as they are equally important for processing the viral replicase polyprotein. Inhibition of either protease blocks the assembly of a functional replication complex. Multiple targets are important because CoV (RNA virus) may mutate to a drug-resistant virus. Combination therapy (such as used for HIV) would reduce the likelihood of selecting for a drug-resistant strain.

18. Line 235: delete maturation, replace with replication

Referee #4:

Shin et al characterize the PLpro-CoV2 protease and compare it to the PLpro-SARS protease using biochemical, structural, biophysical, and cellular studies. The authors report that PLpro CoV2 is more selective for removing ISG15 modifications while PLpro-SARS is more selective for removing ubiquitin modifications. The authors describe the crystal structure of PLpro from CoV2 in complex with murine ISG15, and probe the structural basis of substrate specificity of PLproCoV2 by comparing the structure with SARS PLpro in complex with K48-Ub2. The structural comparison reveals the residues that interact with the hydrophobic patch of the distal Ub are not conserved in PLproCoV2. The authors show that mutating residues of PLpro-CoV2 that make hydrophobic interactions with the substrate results in loss of activity, based on the loss of reactivity of the V66A and F69A proteins towards ISG-propargyl probes. The authors show that a naphthalene containing compound, GRL-0617, inhibits the activity PLpro-CoV2 and supplement experimental findings with various computationally derived molecular models. In cellular studies, PLpro-CoV2 appears to antagonize the immune response by directly regulating ISG15 modification of the IRF3 transcription factor, and by enhancing viral peptide processing, viral replication, and release. Given the current COVID-19 pandemic these studies are timely and of broad reader interest, particularly since PLpro-CoV2 was not included in a recent protein interaction map study (Nature. 2020 Apr 30. doi: 10.1038/s41586-020-2286-9). That said, for these specific reasons it is all the more critical that the authors are very clear about their conclusions, are open about where uncertainty remains, and do not over-state their claims. If the authors are able to address the concerns outlined below, this work would be publishable in Nature.

Fig. 1a: catalytic dead proteases should be included as controls to confirm decreases in ubiquitinated or ISGylated proteins from whole-cell lysates are due to protease activity and not due to artifact, eg. nonspecific proteases or aggregation during reaction incubation. Also the ISG15 blot could be lighter.

Fig. 1b, Ext data Fig. 1b: PLpro-CoV2 appears to react with the K48-Ub2 probe in Fig. 1b but not Ext data Fig. 1b, can the authors explain?

How does PLPro-CoV2 ISG15-probe reactivity and ISG15-AMC reactivity compare with the ISG15 deconjugating enzyme UBP43/USP18?

Probe reactivity isn't necessarily the best strategy to evaluate protease selectivity; in the absence of bona fide substrates the authors should consider using Ub-AMC, ISG15-AMC, and other related UBL substrates to cross-compare their findings (these are commercially available). Alternatively, or better yet additionally, the authors should perform binding studies (eg SPR) to measure the affinities of the purified PLpro enzymes with the various Ub and UBL proteins as relative binding affinities may explain respective SARS protease selectivity. The MD simulations do not fully address this question, particularly since the studies are done with mISG15 (additional comments below).

Fig. 2a: The annotation of the catalytic triad using a single arrow pointing away from the protein is confusing.

Fig. 2b: Please display SARS-CoV2 PLpro in the same orientation as Fig. 2a.

Fig. 2c: Removing the main chain stick rendering would make it easier to discern the sidechains such as Thr75.

Fig. 2d: It would be useful to have lane with ISG15-Prg only and/or a time 0 sec in order to assess how much is ISG15-Prg is being added to each lane and the ratios of modified to unmodified, similar to Extended data Fig. 2b.

The authors show that mutating residues of PLproCoV2 that make hydrophobic interactions with the substrate results in loss of activity, based on the reactivity of the V66A and F69A proteins towards ISG-propargyl probes. It would be useful to know the conservation of the highlighted residues across other PLpro enzymes.

Why did the authors use murine IGS15 instead of the more relevant human IGS15 in their structural studies? The overall structures of murine and human IGS15 are different (Daczkowski et al., 2017 JMB; Dzimianski et al 2019 JMB). A sequence alignment of human and murine IGS15 as well as a discussion of the different structures of human and mouse IGS15 in complex with different coronavirus PLpro enzymes should be included in the extended data.

Fig. 3a: the authors should expand on why GRL-0617 was chosen as the lead inhibitor candidate for PLpro-CoV2. Does the GRL-0617 enantiomer abolish inhibitory function, that would suggest specificity? Were other protease inhibitors considered? Can a similarity search reveal other compounds that may act as more potent inhibitors for PLpro-CoV2, based on the authors' structural data?

Fig. 3b: The authors show the structure of PLproCoV2 in complex with IGS15 next to a model of the structure of PLproCoV2 in complex with GRL-0617. It is interesting that the authors highlight Y268 in both figures, especially since in their crystal structure of PLproCoV2 in complex with mIGS15, Tyr268 is not part of the final crystallographic model and the region of PLproCoV2 protein from residue T265 to H272 has no electron density. The authors should indicate if this region of the protein is derived from a computational model.

Fig. 3c: Using Ub-AMC as the substrate for both PLpro-SARS and PLpro-CoV2 is inconsistent with the conclusion from studies in Fig. 1: "Taken together, these results indicated that PLproCoV2 strongly prefers ISG15 as a cleavage substrate" (lines 87-88), especially since the authors conclude from Fig. 1a that "PLproSARS strongly reduced the smears of ubiquitinated proteins and ISGylation of substrates, whereas PLproCoV2 preferentially reduced ISGylation of protein substrates" (lines 69-70). Instead, the authors should evaluate SARS PLpro inhibitory activity of GRL-0617 using an ISG15-based substrate. Furthermore, the authors use normalized activity to indicate that GRL-0617 no longer inhibits the Y268G mutant, supporting their suggestion that this residue is important for inhibitory activity. However, the scale of the x-axis for the Y268 mutant starts at 200 suggesting the catalytic activity of Y268G is severely perturbed. Again, it is not clear why the Y268G mutant was selected instead of the Y268T mutant to explain the lack of activity if GRL-0617 towards MERS PLpro. The authors should show that the Y268G mutant has comparable catalytic activity to the wildtype.

Fig. 3d: the ISG15 blot is quite dark and the data do not show convincing differences. This experiment should be repeated.

Fig. 3e: using GRL-0617 at up to 400µM is a remarkably high concentration, particularly given the data in Fig. 3c indicating that GRL-0617 is a sub-µM inhibitor. The authors should explain why there is a discrepancy in the data and also need to show that GRL-0617 remains selective at such high concentrations.

Fig. 4a, b: the figure legend and the x-axis of Fig. 4a are not consistent, the authors need to clarify if these are WT or mutant PLpro proteins. If they're mutant, the mutant residues should be specified.

Fig. 4c: did the authors denature lysates prior to IRF3-Myc IP? If not, it is not clear whether the ISG15 IB of IRF3-Myc-associated proteins indicates that ISG15-modified proteins are co-associated with IRF3, or whether IRF3 is covalently ISG15-modified.

Lines 191-193: the authors conclude "our findings indicate how two closely related coronaviruses (SARS and SARS-CoV-2) could differentially counteract the host-immune system by their PLpro enzymes." The differences are really not that great, eg Fig. 4d, 4e, Ext data Fig 4c, 4d, 4e, 4f, 4g, and 4h. Furthermore the same inhibitor appears to affect the two different PLpro proteases similarly: Fig. 3c, Ext data Fig 4g, 4h. Yes CoV2 PLpro appears to be more effective in regulating ISG15 modifications and SARS PLpro is more effective with Ub modifications, but both are quite effective in downregulating IFN β levels with polyI:C treatment (Fig. 4e) and both also regulate TNF-induced NF κ B expression (Fig. 4f). Instead of using the GRL inhibitor, the authors should instead transfect cells with catalytic mutant versions of the PLpro proteins in Fig. 4e and 4f.

Fig. 5c, d, g: again 50-100 μ M is a very high dose of the protease inhibitor GRL-0617. The authors need to demonstrate that this dose is selectively inactivating PLpro-CoV2 and not host or other viral proteases, the inhibition of which may contribute to the noted cellular efficacy.

Lines 330-332: The authors indicate using TALON beads purification with buffers containing 2mM DTT or 1mM TCEP, please confirm that these were the buffer conditions used.

Lines 353-368: The description of the protein concentrations used in the crystallization is confusing and should be clarified with more detail. The authors state the concentration of the complex is 250 μ M, and indicate a 1:1 protein:mother liquor dilution yet state the protein concentration as still being 250 μ M. Are the authors referring to the initial or final concentration? The authors go on to describe optimized crystals using a different protein concentration. Was this different concentration achieved by varying the drop ratios or by diluting the protein? There is no information on murineISG15, the buffer it was in and whether it was modified at the C-term to facilitate a covalent interaction with C111S and whether there was additional gel filtration step after the complex was formed.

In the crystallographic model of PLproCoV2 in complex with ISG15 the hydroxyl from C111S is 2.3 $\text{\AA}$ from the C-terminal carbonyl oxygen of murineISG15, this is too close for what is likely to be an unfavorable interaction.

Lines 401-440: The methods describing the set up for the MD simulation of PLproCoV2 with K48-Ub2 and the MD simulation of PLproCoV2 with mISG15 are different. Since the authors are comparing the results of these two simulations the setup should be as similar as possible, accepting the differences in the starting models.

To generate the starting model of PLproCoV2 with K48-Ub2 for the MD simulation, the authors used coordinates from two different structures, PDB-ID 6W9C for apo-PLproCoV2 and PDB-ID 5E6J for K48-Ub2. However, it is clear from a comparison of crystallographic structures of apo-PLproCoV2 and PLproCoV2 in complex with substrates, as well as structures of other PLpro enzymes, that there is considerable movement around the active site when substrate is bound. Therefore, it seems unsurprising that in the MD simulation using apo-PLproCoV2 as the starting model for a complex with K48-Ub2 superimposed, that the K48-Ub2 moves away from the active site. It would be more appropriate to use the coordinates of PLproCoV2 taken from a substrate complex, or use a homology model based on a substrate complex as the starting model for the MD simulation.

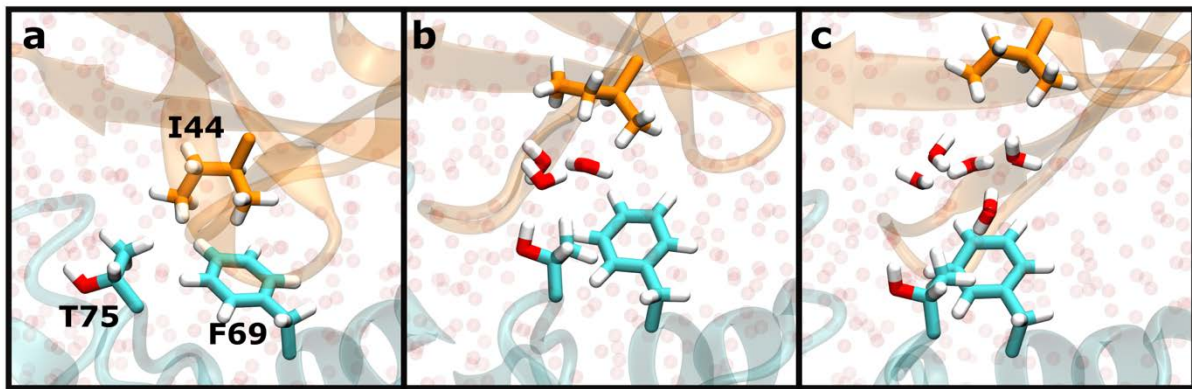
Author Rebuttals to Initial Comments:**Referee #1 (Remarks to the Author):**

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To see whether the cause of the specificity differences lies perhaps in the dynamics of the interactions, the authors performed molecular dynamics simulations. However, in my view, these did not reveal additional findings concerning the differences in specificity.

We thank the reviewer for raising this issue. We agree that molecular dynamics simulations give us a unique opportunity to explore the origin of the differences in specificity. In response, we carefully inspected our original simulations and performed new simulations, including for mutants of SARS^{PLpro} to gain insight into the molecular factors determining specificity. The disruption of a hydrophobic triad in the core of the K48-Ub₂ binding interface emerges as the main factor, primarily due to the mutation of Leu76^{SARS} to Thr75^{CoV2}. In the revised manuscript, we include the results of the new simulations in a detailed discussion of the dissociation mechanism:

“Multi-microsecond molecular dynamics (MD) simulations confirmed that CoV2^{PLpro} interacts more tightly with ISG15 than with K48-Ub₂ (Extended data Fig. 2d, e). In three independent runs of 3.2 μs each, mISG15 remained bound as in the CoV2^{PLpro}:mISG15 X-ray structure. By contrast, the distal ubiquitin of K48-Ub₂ separated from CoV2^{PLpro} in four out of six runs on a microsecond time scale. As the primary cause, we identified the L75T mutation in CoV2^{PLpro}, which weakens the hydrophobic cluster in the binding interface. Water transiently entered between Ile44^{Ub} and Thr75^{CoV2} on pathway to dissociation. Indeed, in simulations of the S67V/L76T CoV2^{PLpro} double mutant with K84-Ub₂ bound, the distal ubiquitin transiently dissociated by the same mechanism (Extended data Fig. 2f, 2g).”



(Extended data Fig. 2g, Dissociation pathway of distal Ub from CoV2^{PLpro}.)

a) Initial hydrophobic interactions between F69(CoV2), T75(CoV2) and I44(Ub). b) Water wedges in between T75(CoV2) and I44(Ub). c) Water penetration between T75(CoV2)/F69 (CoV2) and I44(Ub) leads to dissociation.

The authors went on to test the compound GRL0617, which is a proven inhibitor for the SARS-CoV PLpro, and found that it also efficiently inhibits SARS-CoV-2 PLpro.

Next, Shin et al. investigated the interactomes of SARS-CoV-2 PLpro and SARS-CoV PLpro. This revealed that the enzyme of the novel coronavirus mainly affects signaling proteins involved in the control of interferon production, whereas SARS-CoV PLpro interfered with proteins of the NFkappaB pathway that mainly controls cytokine production.

This manuscript presents very interesting data. Unfortunately, the differences between the substrate specificities of these highly similar proteins are not readily explained by comparing their three-dimensional structures. Yet, the findings are of very high importance and relevance.

Individual concerns:

(1) It is not clear whether the authors work with the full-length SARS-CoV-2 PLpro or cut off the N-terminal ubiquitin-like domain (Ubl), as it is often done when the full-length enzyme refuses to crystallize.

For entire study we used construct that contains PLpro and Ubl domain of Nsp3. It is mentioned now in the plasmid construction section in Material and Methods.

“Protein sequence for CoV2^{PLpro}-Ubl domain (amino acids, 746-1060) of Nsp3 protein from SARS-CoV-2 (Nsp3; [YP_009725299.1](#)) was codon optimized, synthesized and cloned into pET28b with NcoI and XhoI to have C-terminal His-tag (Genescript). Protein sequences of PLpro-Ubl domain of SARS and MERS from (PDB ID: 3MJ5, 5W8U, respectively) were also codon optimized, synthesized and cloned into pET28b with NcoI and XhoI to have C-terminal His-tag (Genescript).”

(2) line 50: "mortality" should be replaced by "case - fatality rate". "Mortality" is the fraction of the total population succumbing to the viral infection and is extremely low. "Case - fatality rate" is the fraction of diseased people who die from the infection.

Thanks for the clarification and we have changed the mortality to case-fatality-rate as suggested.

(3) line 52: The MERS outbreak of 2012 cannot be called "global". The virus only surfaced on the Arab peninsula and, in 2015, there was a travel-related outbreak in South Korea. It should also be mentioned that the MERS-CoV outbreak on the Arab peninsula is still ongoing.

We agree with this comment and removed “global” from the text.

(4) line 55: "a critical step" or what? Something missing here.

We have removed the “a critical for” from the sentence and re-wrote the sentences.

(5) line 95: reference missing for MERS-CoV PLpro in complex with human ISG15.

The PDB code (PDB ID: 6BI8) for MERS-CoV PLpro complex is inserted. There is no publication for this structure.

(6) line 97: reference missing for crystal structure of ISG15 on its own.

The PDB code (PDB ID: 5TLA) and reference for ISG15 is inserted.

(7) line 99: reference missing for SARS-CoV PLpro in complex with C-terminal domain of ISG15 (do not provide only PDB code)

The reference for the complex structure of SARS-CoV PLpro with C-terminal domain of ISG15 is inserted.

(8) line 181: reference missing

Corrected.

(9) lines 182, 189: IkappaB

We have replaced the k to κ .

(10) line 190: NF-kappaB

We have replaced the k to κ .

(11) lines 317, 318, 404, 615, 618, 619, 624: references for crystal structures missing (do not only provide PDB codes!).

We have provided the references for all structures except the 6W9C which does not have linked publication.

(12) line 354: Equal amounts ...

Corrected.

(13) lines 381, 382, 386: ... as previously described

Corrected.

(14) line 383: purified by size-exclusion chromatography

Corrected.

(15) line 386: CSN should be explained upon first occurrence.

The Cop9 signalosome is inserted.

(16) line 404, 415, 424: What is the apo form of the PLpro? An apoenzyme is an enzyme that lacks an essential cofactor. In case of the PLpro, the apoenzyme would be the protein lacking the zinc ion bound to the fingers domain. With all components present, the enzyme would be called a holoenzyme. However, this is not what the authors mean. Unfortunately, the term "apo" is being widely misused by structural biologists to indicate the absence of the substrate of an enzyme. (What would be the holoenzyme then?). It is the obligation of authors and editors to keep the language of science as clean and unambiguous as possible and therefore, the incorrect usage of the term "apo"

should be banned.

We thank to reviewer for this specific correction, we have changed the wording for referring structures in the manuscript. We changed the apo form to substrate unbound structure.

(17) line 418: Is this the BL2 loop?

We thank reviewer for this specific correction. We now introduce the loop as blocking loop 2 (BL2 loop).

(19) line 428: I am not sure about the catalytic His being protonated. This would assume that the active-site Cys is negatively charged. This is true for papain but has, to the best of my knowledge, not been demonstrated for coronavirus PLpros.

We agree with the reviewer that His272 protonation is an interesting question. In response, we performed an additional simulation of CoV2^{PLpro} with a neutral active-site His(272). In a ~600 ns long simulation, the active site structure remained stable, similar to the simulation with His(272) protonated. However, the local charge imbalance in the active site with a neutral His(272), its direct interaction partner Asp(286), as well as Glu(263) and Lys(274), transiently attracted sodium ions into the immediate vicinity. We also performed additional pKa calculations using the H++ web server (<http://biophysics.cs.vt.edu/>) using the 6w9c re-refined structure. These calculations gave a pKa of 7.2 for His(272). Based on the results of the MD simulations in different protonation states and of the pKa calculations we cannot exclude entirely that His(272) is in an equilibrium between the charged and a neutral form. We now point this out in the paper in line 428:

“We cannot exclude that the protonation state of the catalytic His272^{CoV2} (His273^{SARS}) is in equilibrium between charged and neutral forms.”

Importantly, considering the large distance and the structural stability irrespective of protonation state observed in our simulations, we expect that the active site protonation state does not impact the interaction with the distal substrate.

(20) line 624: Why is the PLpro called PLP2 here?

Corrected.

(21) Reference 11: Something seems to have gone wrong here.

Corrected.

(22) The table with crystallographic data is not readable, in parts.

Corrected.

Referee #2 (Remarks to the Author):

In “Inhibition of papain-like protease PLpro blocks SARS-CoV-2 spread and promotes viral entry”, Shin et al. characterize the host-directed effects of PLpro from SARS-CoV-2. Strengths of this study are the frequent and thorough comparisons of PLpro from SARS-CoV and SARS-CoV-2, thorough structural and biochemical characterization of PLpro, and the use of functional assays to demonstrate the relevance of their proposed mechanism during infection in cells. The major concern is novelty. The crystal structure of a CoV PLP in complex with ISG15 has been reported (PMID: 28438633), and GRL0617 is well characterized as inhibiting CoV replication by targeting PLP (PMID: 18852458). Thus, the major findings of this study are largely expected based on the high similarities between SARS-CoV-2 and SARS-CoV. The authors do reports some notable distinctions between the two PLPs which are very interesting and add new insight into the distinct biology of these viruses. If the authors demonstrated antiviral activity of GRL0617 against SARS-CoV-2 in more relevant systems, this would likely significantly improve the broad interest in this study. Additionally, some of the main conclusions would benefit from additional supporting evidence, as outlined below.

Major comments

1. Related to figure 2C: The authors suggest based on structural data and modelling that PLproSARS L76 interacts with ubiquitin I44, whereas PLproCoV2 T75 does, perhaps explaining the differences in substrate specificity. It should be easy to introduce reciprocal mutations into the two proteases and test this prediction functionally, especially since the authors test LOF mutations for the CoV-2 protease in 2D. Functional data would greatly enhance this conclusion.

We thank the reviewer for asking this specific question. With this additional experiment our conclusion of substrate specificity is greatly enhanced and enabled us to come to better understanding of activity of both SARS and CoV2 PLpros in cells. As the reviewer suggested, we have introduced the T75L mutation into SARS-CoV2 PLpro and tested its activity on both K48-Ub₂-AMC and ISG15-AMC and added new figures (Figure 2d). The T75L mutant significantly rescued the CoV2^{PLpro} activity on K48-Ub₂-AMC while the activity on ISG15-AMC remained similar. It suggests that CoV2^{PLpro} gets its ISG15 preferences by weakening its interaction to ubiquitin which is also consistent with our new binding kinetics data (Figure 1e). To validate this further we also checked the ISG15-AMC processing rate in the presence of non-hydrolysable triazole K48-Ub₂ (Extended data Figure 1c). While triazole K48-Ub₂ inhibits SARS^{PLpro} activity on ISG15-AMC, this inhibitory effect was not observed on CoV2^{PLpro}. In addition, MD simulation with SARS^{PLpro} double mutants (S67V/L76T) further confirmed the transient release of distal ubiquitin of K48-Ub₂ (Extended data Figure 2f).

2. The authors observe that PLpro SARS associates with SERPINs while PLproCoV-2 does not and note that SERPIN family proteins are known to inhibit papain-like protease activity. This could be another compelling difference between PLproSARS and PLproCoV-2 if the latter has somehow adapted to avoid potential inhibition by SERPINs. Functional assays could address this and potentially enhance the novelty of this study.

We thank the reviewer for raising this question. To gain functional importance of differential binding affinities of SERPINs to PLpros, both PLpro and SERPIN-B3 were co-expressed in the A549 cells. Consistent with mass spectrometry results, expression of SERPIN significantly rescued the NF- κ B expression from SARS^{PLpro} expressing cells, but not from CoV2^{PLpro} expression cells. The new data is presented in Extended data Figure 5 a, b. This is an evidence for validation of presented proteomic data but more functional studies are needed to better understand the importance of global interactome differences between SARS and CoV2 PLpros. These questions are planned for follow-up studies.

3. Figure 5d: The authors quantify viral RNA in supernatant as a readout for viral production. The gold standard assays for virus production are those that discriminate between defective particles and truly infectious particles, such as plaque assays and TCID50 assays. Such an assay would complement the existing data.

We thank the reviewer pointing to this issue: Since the PCR in the originally submitted version was detecting full length genomic RNA only that might originate from defective viruses, we now additionally quantify an mRNA isoform that correlates with the production of infectious particles into the supernatant (Figure 5c). Only full-length genomic RNA is packaged into newly assembled virions, so we had to measure active replication within the infected cells, which is an accepted method in the field (Wölfel et al. 2020, Nature, 581(7809):465-469. We measured SARS-CoV-2 sgRNA4 encoding E gene, which is processed during discontinuous transcription only in cells with active replication (Kim et al. 2020, Cell, doi: 10.1016/j.cell.2020.04.011.). Thus, we are now able to discriminate between defective particles and truly infectious particles produced in the infected cell. We also added the corresponding references to the manuscript and explained this assay in the methods section.

4. Figure 5e: It appears that GRL-0671 reduces the detection of free ISG15 in the IP in the absence of SARS-CoV-2 infection. Could the authors comment on this? Is it possible that GRL-0671 is impacting either loose association of ISG15 with IRF3 or ISG15 levels at basal states? Probing the input membrane for ISG15 would be helpful, as would an isotype control to demonstrate the specificity of the IP.

We have not observed consistent effect of GRL on the level of ISG15. We repeated experiments and new data is presented in Figure 5e showing that there is no significant effect of GRL. As reviewer #3 suggested, we also included SARS infection data in the revised manuscript in the same assays.

5. It is unclear why the authors invoke discussion of the DNA sensing pathway in Lines 169-172. CoV are RNA viruses. They would not activate innate immunity through cGAS-STING-IRF3, but rather through MAVS-IRF3. And the Plos One paper (ref 33) claiming PLP antagonism of STING did not confirm this with live virus. It should thus be taken with a grain of salt. This section should be edited to reflect more accurately the known CoV sensing pathways.

We fully agree with the reviewer on this comment and we have thus removed these comments. The proteomic analysis of WT versus catalytic mutant of CoV2^{PLpro} is preliminary and it did not contribute any new information. We thus removed it completely and rather added more quantification and validation to the proteomic analysis of the interactome difference between SARS and CoV2 PLpros.

Minor points & typographical errors

line 55 – “a critical xx for” (missing word)

Corrected.

line 181 – “[ref]” missing

Corrected.

line 185 – Viral “entry” is not mimicked by poly(I:C) treatment – sensing of viral nucleic acid is mimicked by this assay.

Corrected.

Figure 4b is not explicitly discussed in the text.

We have removed this figure in the revised manuscript.

Line 232 – the study does not necessarily establish that PLpro is “an equally promising target” as 3CLpro, as no comparisons are made within this text. It would be more appropriate to simply state that PLpro is “an additional promising target” or something along those lines.

Corrected.

Referee #3 (Remarks to the Author):

This manuscript by Shin and co-workers describes an impressive array of biochemical assays, structural studies, and pharmacological studies that document the multifunctional role of the papain-like protease (PLpro) during SARS-CoV-2 replication. These studies leveraged 15 years of

previous work, but instead of acknowledging the previous work in the background section of the paper, the authors instead selectively incorporate the previous studies into the presentation of their own experiments, which implies that the previous studies support their results, instead of the other way around. The authors should re-write this paper in a way that conveys to other researchers around the world how previous studies that documented the multifunctional nature of all coronavirus papain-like proteases, structural studies that identified residues that interact with ubiquitin, screening of libraries to identify non-covalent inhibitors, and co-crystal structural studies that identified the site bound the inhibitor, can all be leveraged to make rapid progress toward developing therapeutics that inhibit the replication of SARS-CoV-2, and have potential for treating COVID-19 patients.

Comments for the authors' consideration:

1. Abstract: CoV proteases process the viral polyprotein to generate a functional replicase complex. It is not clear what is meant by viral maturation, so this should be deleted.

We have corrected the sentence as suggested.

2. Abbreviations: The typical presentation is to name the virus first, and then the enzyme, so SC-PLpro and SC2-PLpro would be more appropriate than the current superscript designation.

We thank the reviewer for this suggestion and we now named the virus first and then the PLpro. (e.g SARS^{PLpro} and CoV2^{PLpro})

3. Abstract: Would it be accurate to state that it was by comparing previous structural information on binding of GRL-0617 into a flexible loop adjacent to the PLpro active site, to the new structural informational on SC2-PLpro which documents the same flexible loop adjacent to the active site, that suggested that the previously identified inhibitor would block the active site of both enzymes? It is not clear why you would need to leverage the SC2-PLpro-ISG15 structure to appreciate the similarities of the two enzymes and the presence of the flexible loop.

We agree and thank the reviewer for this suggestion. It was indeed not correctly represented due to space limitation in the first abstract. In the revised manuscript we had no space to mention GRL within abstract but we have made all efforts in the text to properly describe how we landed on the GRL-0617 by referring the previous studies of SARS PLpro with naphthalene based compounds from Andrew Mesecar's lab [Ratia, et al, 2008, PNAS, Ghosh, et al, 2010, J. Med. Chem, Baez-Santos, et al, 2014, J. Med. Chem.].

4. Background section: Comparing the recent progress on 3CLpro to PLpro is not really the important issue here. Instead focus on how leveraging previous studies of PLpro allows researchers to make rapid progress on identifying inhibitors of SARS-CoV-2 PLpro.

We agree on this topic as well and we have now discussed more on studies on PLpro with relevant references (ref. 6-8)

5. Line 56: remove maturation, release and further spread, as this is not the function of these proteases.

We have corrected the sentence.

6. Line 59-62, reference the studies that document CoV PLpro multifunctionality and inhibition of the innate immune signaling, not reviews on general innate immunity (ref 9-12).

We have corrected the references.

7. Line 70 and Figure 1. The teeter-totter diagram make it look like it is all or none, but the results indicate the multifunctional nature of both enzymes, with this expressed region of SC2-PLpro exhibiting more robust de-ISGylating activity. The authors should note that PLpro is part of the larger nonstructural protein 3 (nsp3). The context of PLpro within nsp3 may further influence enzyme activity.

We thank the reviewer for these comments. With the new binding kinetics data suggested by reviewer #4, we now changed the diagram to be more graded (Figure 1f). We agree with the comment about the importance of localization of PLpro within Nsp3 protein that is anchored within ER. We have mentioned this in the text. In addition, we have compared also the effect of CoV2^{PLpro} on deISGylation of IRF3 substrates also upon SARS-CoV-2 infection in cells (Figure 5e).

8. Line 125: Provide more information on how GRL-0617 was identified by high through-put screening and SAR modification to help the readers understand how this antiviral was identified.

We thank the reviewer for asking this suggestion. We also apologize that this has not come out clear in the first version. Now we included more information for GRL-0617 identification.

“With the pressing urgency to identify novel therapeutic strategies for COVID-19, we tested GRL-0617, a non-covalent inhibitor of SARS^{PLpro} for its effect on CoV2^{PLpro} (Fig. 3a)¹⁶. The GRL-0617 is a naphthalene-based inhibitor developed against SARS^{PLpro} that does not inhibit other host proteases (USP2, USP7, USP8, USP18, USP20, USP21, USP28, UCH-L1, UCH-L3, DEN1, caspase 3 and cathepsin K)^{16,17}. Based on the binding mode of GRL-0617 or other known naphthalene inhibitors of SARS^{PLpro}16-19,....”

We also included relevant references for naphthalene-based inhibitors developed for SARS PLpro from Andrew Mesecar's lab [Ratia, et al, 2008, PNAS, Ghosh, et al, 2009, J. Med. Chem. Ghosh, et al, 2010, J. Med. Chem, Baez-Santos, et al, 2014, J. Med. Chem.].

Among them, only GRL-0617 is commercially available and we could not get other derivatives.

9. Line 132: Reference the lack of activity of this type of inhibitor for MERS-PLpro (Kilianski et al., 2014), as this is already known.

We thank the reviewer for this information and it is corrected. Concerning this point, the novelty in our work is the identification of Y256 as a major difference between SARS-CoV-2 and MERS. Mutation of this single amino acid blocks the inhibitory effect of GRL-0617

10. Lines 145-147: These results confirm and extend the previous studies that documented that GRL-0617 is an effective inhibitor for SARS-CoV, suggesting that it may be more broadly effective as an inhibitor to the many SARS-related CoVs that are known to exist in bat populations around the world.

We thank the reviewer and we have corrected the sentences.

11. Line 185-193 and Figs 4e and 4f. These overexpression assays and stimulation by poly(I:C) and TNF-alpha may not reflect anything about the biological function of PLpro in the context of virus replication, so should be deleted. During virus replication, PLpro is part of a membrane-associated complex, not free in the cell, so the activity reflected in this assay may or may not reflect the activity of the enzyme in the context of the membrane-associated viral replication complex.

We agree with the reviewer that both the poly(I:C) treatment and PLpro expression cannot reflect the virus infection or replication. Nevertheless, overexpression of CoV2^{PLpro} and SARS^{PLpro} provided us valuable and insightful results including their ability to deubiquitinate and deISGylate of cellular proteins as well as identifying different interacting partners from the cells. We have also repeated the experiment with catalytic dead mutants as reviewer #4 suggested and moved the entire data to Extended data Figure 6. We have now included more detailed studies in primary virus infected cells and our observations with transfected proteins are correlated with cells infected with primary SARS and SARS-CoV-2 viruses.

12. Line 205: remove maturation and release. Inhibiting the protease stops the assembly of the viral replication complex, which is necessary for on-going viral RNA synthesis. If there is no RNA synthesis, then the production of infectious progeny virus stops.

Corrected.

13. Fig 5e. Excellent experiment and clear result. It would be even better if you included infection

with SARS-CoV for a direct comparison with SARS-CoV-2. You may see a very similar result. This experiment would help to determine if the differences you see in substrate preferences in the overexpression studies are also reflected in the activity of the enzyme when it is part of the replication complex.

We thank the reviewer for raising this question. We were fortunate to have also primary SARS isolates in our virology department and could perform this insightful experiment. As presented in Fig 5 d and f infection of epithelial cells with SARS versus SARS-CoV-2 and corresponding treatment with GRL-0617 indicated differences in regulation of IRF3 ISGylation and IRF3 phosphorylation and also weak differences on p65 phosphorylation, as well as ISG15 gene regulation, but not on IL-6 or IL-8 cytokine expression. These are quite impressive results and in accordance with a recent study on differences between SARS-CoV-2 versus others respiratory viruses (Influenza, SARS and MERS) (Blanco-Melo et al, Cell 2020). Our studies strongly advocate that blocking CoV2^{PLpro} enzyme activity with GRL-0617 apart from inhibiting viral replication, promotes interferon responses with a slight increase in pro-inflammatory chemokines and cytokines in the epithelia, the prime site of pathogen entry would mount a strong anti-viral response at the early stage of the infection process. Yet, we also indicate that more detailed studies are needed to better understand the multiple factors that control innate and adaptive immunity to manifest distinct pathologic outcomes of SARS and COVID-19 infections in a cell type and stage specific manner. We thank once again the reviewer for these suggestions.

14. Line 217: blocking processing and assembly of the replication complex and viral RNA synthesis (not virus release)

Thank you. This was corrected.

15. Lines 219-228. This is speculation that PLpro is a differential virulence factor among CoVs, and Niemeyer et al., 2018 PLoS Pathogens should be cited here. It should also be noted that there may be many virulence factors that contribute to the high transmissibility of SARS-CoV-2 and distinct pathologic outcomes (SARS-CoV-2 has a lower case fatality rate than SARS-CoV, which was 10%). Multiple factors contribute to the ability of SARS-CoV-2 to cause a pandemic that differs in transmissibility (higher than SARS-CoV) and morbidity (lower than SARS-CoV).

We agree with the reviewer to include this information and have adjusted our discussion to reflect them including relevant references.

16. Fig 5h. MDA-5 is the PRR for CoVs, not RIG-I; also the right hand side should point to the double membrane vesicles where nsp3 resides, not to virus particles.

Thank you. This was corrected.

17. Line 229-234 – It was already established for SARS-CoV that PLpro is a target for therapeutics. Delete the comparison of 3CLpro (Mpro) and PLpro, as they are equally important for processing the viral replicase polyprotein. Inhibition of either protease blocks the assembly of a functional

replication complex. Multiple targets are important because CoV (RNA virus) may mutate to a drug-resistant virus. Combination therapy (such as used for HIV) would reduce the likelihood of selecting for a drug-resistant strain.

We thank the reviewer for these suggestions and have corrected discussion accordingly

18. Line 235: delete maturation, replace with replication

We have corrected discussion as reviewer suggested.

Referee #4 (Remarks to the Author):

Shin et al characterize the PLpro-CoV2 protease and compare it to the PLpro-SARS protease using biochemical, structural, biophysical, and cellular studies. The authors report that PLpro CoV2 is more selective for removing ISG15 modifications while PLpro-SARS is more selective for removing ubiquitin modifications. The authors describe the crystal structure of PLpro from CoV2 in complex with murine ISG15, and probe the structural basis of substrate specificity of PLproCoV2 by comparing the structure with SARS PLpro in complex with K48-Ub2. The structural comparison reveals the residues that interact with the hydrophobic patch of the distal Ub are not conserved in PLproCoV2. The authors show that mutating residues of PLpro-CoV2 that make hydrophobic interactions with the substrate results in loss of activity, based on the loss of reactivity of the V66A and F69A proteins towards ISG-propargyl probes. The authors show that a naphthalene containing compound, GRL-0617, inhibits the activity PLpro-CoV2 and supplement experimental findings with various computationally derived molecular models. In cellular studies, PLpro-CoV2 appears to antagonize the immune response by directly regulating ISG15 modification of the IRF3 transcription factor, and by enhancing viral peptide processing, viral replication, and release. Given the current COVID-19 pandemic these studies are timely and of broad reader interest, particularly since PLpro-CoV2 was not included in a recent protein interaction map study (Nature. 2020 Apr 30. doi: 10.1038/s41586-020-2286-9). That said, for these specific reasons it is all the more critical that the authors are very clear about their conclusions, are open about where uncertainty remains, and do not over-state their claims. If the authors are able to address the concerns outlined below, this work would be publishable in Nature.

Fig. 1a: catalytic dead proteases should be included as controls to confirm decreases in ubiquitinated or ISGylated proteins from whole-cell lysates are due to protease activity and not due to artifact, eg. nonspecific proteases or aggregation during reaction incubation. Also the ISG15 blot could be lighter. We have repeated experiment with the catalytic dead proteases (C111S mutant) and replaced the Figure 1a.

Fig. 1b, Ext data Fig. 1b: PLpro-CoV2 appears to react with the K48-Ub2 probe in Fig. 1b but not Ext data Fig. 1b, can the authors explain?

Because of the strong reactivity of propargyl warhead, we designed and performed the assay on the ice with very short time points. Under this optimized condition, we nicely observed the different substrate specificities of two PLpros. We used different experimental setup for Figure 1b (shortest time point: 15 seconds) and Ext. data Fig. 1b (5 seconds) and now we added the condition in the Ext. data Fig. 1b.

How does PLPro-CoV2 ISG15-probe reactivity and ISG15-AMC reactivity compare with the ISG15 deconjugating enzyme UBP43/USP18?

We thank the reviewer for raising this question. We have tested the reactivity of mUSP18 with ISG15-Propargyl probe and ISG15-AMC and added new figures (Extended Figure 1e, 1f). While USP18 and CoV2^{PLpro} showed similar activity to propargyl-based probe, CoV2^{PLpro} showed better catalytic efficiency on ISG15-AMC cleavage.

Probe reactivity isn't necessarily the best strategy to evaluate protease selectivity; in the absence of bona fide substrates the authors should consider using Ub-AMC, ISG15-AMC, and other related UBL substrates to cross-compare their findings (these are commercially available). Alternatively, or better yet additionally, the authors should perform binding studies (eg SPR) to measure the affinities of the purified PLpro enzymes with the various Ub and UBL proteins as relative binding affinities may explain respective SARS protease selectivity. The MD simulations do not fully address this question, particularly since the studies are done with mISG15 (additional comments below).

We thank the reviewer for raising this question and agree with that the propargyl reactivity is not the best strategy to see the cleavage activity of proteases. As the reviewer suggested, we tested the activity of both PLpro with K48-Ub₂-AMC or ISG15-AMC and determined the kinetics (Figure 1d, Extended data Table 2). CoV2^{PLpro} showed 2-fold better catalytic efficiency (k_{app} , k_{cat}/K_M) to ISG15-AMC than K48-Ub₂-AMC, while SARS^{PLpro} showed better catalytic activity on K48-Ub₂. We also performed Bio-layer interferometry (BLI) to measure the binding kinetics of K48-Ub₂ and ISG15 (human / mouse) to both PLpro catalytic dead mutants (C111S). CoV2^{PLpro} had significantly lower dissociation constant (Kd) for ISG15 than K48 Ub₂, while SARS^{PLpro} showed the highest affinity for K48-linked ubiquitin chains (Figure 1e, Extended data Table 3).

Fig. 2a: The annotation of the catalytic triad using a single arrow pointing away from the protein is confusing.

Thank you. This was corrected.

Fig. 2b: Please display SARS-CoV2 PLpro in the same orientation as Fig. 2a.

Thank you. This was corrected.

Fig. 2c: Removing the main chain stick rendering would make it easier to discern the sidechains such as Thr75.

Thank you. This was corrected.

Fig. 2d: It would be useful to have lane with ISG15-Prg only and/or a time 0 sec in order to assess how much is ISG15-Prg is being added to each lane and the ratios of modified to unmodified, similar to Extended data Fig. 2b.

We have repeated the experiment and replaced the figure as reviewer suggested.

The authors show that mutating residues of PLproCoV2 that make hydrophobic interactions with the substrate results in loss of activity, based on the reactivity of the V66A and F69A proteins towards ISG-propargyl probes. It would be useful to know the conservation of the highlighted residues across other PLpro enzymes.

We have compared the PLpro from different coronaviruses including human coronaviruses (229E, NL63, OC43). The sequence alignment is included in Extended data Figure 3.

Why did the authors use murine ISG15 instead of the more relevant human ISG15 in their structural studies? The overall structures of murine and human ISG15 are different (Daczkowski et al., 2017 JMB; Dzimianski et al 2019 JMB). A sequence alignment of human and murine ISG15 as well as a discussion of the different structures of human and mouse ISG15 in complex with different coronavirus PLpro enzymes should be included in the extended data.

We thank the reviewer for raising this question and agree with that there are differences between ISG15 from different species. For our crystallization trials, we also tried with human ISG15 and got crystals but it diffracted poorly and we could not solve the structure with them. In the revised manuscript, we present binding kinetics of mouse ISG15 and human ISG15 to SARS-CoV2 PLpro and there are no significant differences between them (Figure 1e). It is also consistent with recent publication. Freitas et al, nicely tested the SARS-CoV-2 PLpro activity toward ISG15 from different species (<https://pubs.acs.org/doi/10.1021/acsinfecdis.0c00168>, figure attached from their paper) and showed that CoV2 PLpro can process both human and mouse ISG15.

[redacted]

The structural comparison of human ISG15 bound MERS^{PLpro} with our mouse ISG15 bound CoV2PLpro is presented in Extended data Fig. 2a showing the PLpro bound ISG15 conformation is indeed similar to each other which is consistent with our binding kinetics data (Fig. 1e) and above references. As reviewer suggested, we have also included sequence alignment of human and mouse ISG15 in the Extend data Fig 2a)

Fig. 3a: the authors should expand on why GRL-0617 was chosen as the lead inhibitor candidate for PLpro-CoV2. Does the GRL-0617 enantiomer abolish inhibitory function, that would suggest

specificity? Were other protease inhibitors considered? Can a similarity search reveal other compounds that may act as more potent inhibitors for PLpro-CoV2, based on the authors' structural data?

We thank the reviewer for this question. We chose GRL-0617 based on the previous knowledge on naphthalene-based inhibitors developed for SARS PLpro from Andrew Mesecar's lab [Ratia, et al, 2008, PNAS, Ghosh, et al, 2010, J. Med. Chem, Baez-Santos, et al, 2014, J. Med. Chem.]. Among them, only GRL-0617 is commercially available and we could not get other derivatives. Since other inhibitors developed later (3k, 3j, 3e and 5c, Baez-Santos, et al. 2014, J. Med. Chem.) showed lower IC₅₀ value one should test these compounds on SARS-CoV-2 in future. We also cite three new DOI manuscripts describing a potentially new type of inhibitors of CoV2^{PLpro} that have not been yet tested in infected cells with primary SARS-CoV-2.

Fig. 3b: The authors show the structure of PLproCoV2 in complex with IGS15 next to a model of the structure of PLproCoV2 in complex with GRL-0617. It is interesting that the authors highlight Y268 in both figures, especially since in their crystal structure of PLproCoV2 in complex with mIGS15, Tyr268 is not part of the final crystallographic model and the region of PLproCoV2 protein from residue T265 to H272 has no electron density. The authors should indicate if this region of the protein is derived from a computational model.

We thank the reviewer for this specific correction and we clarified that both structures are computational models. (Figure 3 legends)

Fig. 3c: Using Ub-AMC as the substrate for both PLpro-SARS and PLpro-CoV2 is inconsistent with the conclusion from studies in Fig. 1: "Taken together, these results indicated that PLproCoV2 strongly prefers ISG15 as a cleavage substrate" (lines 87-88), especially since the authors conclude from Fig. 1a that "PLproSARS strongly reduced the smears of ubiquitinated proteins and ISGylation of substrates, whereas PLproCoV2 preferentially reduced ISGylation of protein substrates" (lines 69-70). Instead, the authors should evaluate SARS PLpro inhibitory activity of GRL-0617 using an ISG15-based substrate. Furthermore, the authors use normalized activity to indicate that GRL-0617 no longer inhibits the Y268G mutant, supporting their suggestion that this residue is important for inhibitory activity. However, the scale of the x-axis for the Y268 mutant starts at 200 suggesting the catalytic activity of Y268G is severely perturbed. Again, it is not clear why the Y268G mutant was selected instead of the Y268T mutant to explain the lack of activity if GRL-0617 towards MERS PLpro. The authors should show that the Y268G mutant has comparable catalytic activity to the wildtype.

We thank the reviewer for raising these valid points. We have tested the activity of GRL-0617 on ISG15-AMC and the new figure is now presented (Figure 3c). We also generated Y268T mutant as the reviewer suggested and tested on ISG-AMC (Fig. 3c).

Fig. 3d: the ISG15 blot is quite dark and the data do not show convincing differences. This experiment should be repeated.

We inserted short exposure blots. We observed similar differences in deISGylation between SARS and CoV-2 and are confident in these findings. The new data on primary infected cells also indicate differences in de ISGylation of endogenous protein substrates between these two viruses.

Fig. 3e: using GRL-0617 at up to 400 μ M is a remarkably high concentration, particularly given the data in Fig. 3c indicating that GRL-0617 is a sub- μ M inhibitor. The authors should explain why there is a discrepancy in the data and also need to show that GRL-0617 remains selective at such high concentrations.

We thank the reviewer for raising this point and we agree with that 400 μ M is a high concentration but we never used it in any of the other assays particularly not in cells. We used this high concentration in vitro because of the strong reactivity of the propargyl warhead. The specificity of GRL-0617 has been already tested against a panel of DUBs showing specificity for SARS, and not MERS or other human DUBs ([Baez-Santos, et al, J. Med. Chem. 2014](#), [Ratia, et al, PNAS, 2008](#)).

Fig. 4a, b: the figure legend and the x-axis of Fig. 4a are not consistent, the authors need to clarify if these are WT or mutant PLpro proteins. If they're mutant, the mutant residues should be specified. We thank the reviewer for this specific correction. We have corrected the legend and specified the mutant in the axes.

Fig. 4c: did the authors denature lysates prior to IRF3-Myc IP? If not, it is not clear whether the ISG15 IB of IRF3-Myc-associated proteins indicates that ISG15-modified proteins are co-associated with IRF3, or whether IRF3 is covalently ISG15-modified.

Since the IP was done in 1% TritonX (no SDS)- we cannot rule out interaction of other ISGylated proteins with IRF3. We have mentioned this in the text.

Lines 191-193: the authors conclude "our findings indicate how two closely related coronaviruses (SARS and SARS-CoV-2) could differentially counteract the host-immune system by their PLpro enzymes." The differences are really not that great, eg Fig. 4d, 4e, Ext data Fig 4c, 4d, 4e, 4f, 4g, and 4h. Furthermore the same inhibitor appears to affect the two different PLpro proteases similarly: Fig. 3c, Ext data Fig 4g, 4h. Yes CoV2 PLpro appears to be more effective in regulating ISG15 modifications and SARS PLpro is more effective with Ub modifications, but both are quite effective in downregulating IFN β levels with polyI:C treatment (Fig. 4e) and both also regulate TNF-induced NF κ B expression (Fig. 4f). Instead of using the GRL inhibitor, the authors should instead transfect cells with catalytic mutant versions of the PLpro proteins in Fig. 4e and 4f.

We thank the reviewer for these comments. We have now included the data for catalytic mutants. The data is now moved to extended data Figure 6.

Both PLpros work in similar pathways but there are fine and reproducible (from in vitro to in vivo data) differences in the extent of regulation of the 2 pathways (IFN and NF- κ B). We provide now clear differences in biochemical and biophysical parameters particularly in binding and cleaving

ubiquitin chains, and less on ISG15 (Fig 1, Fig. 2 c,d,e,f, Fig 3d) as well as in cells transfected with these proteases (Fig. 4). Though the differences are small they are significant, and can be relevant in infected cells. We also agree with reviewer 3 that these data require careful and balance interpretation, as the native PLpros are part of an ER-anchored nsp3 protein, that may influence in vivo activities of these enzymes and influence on innate and adaptive immunity. Thus, we are happy to now provide also comparison between SARS and SARS-CoV-2 infected cells where we observe stronger impact of CoV-2 on IFN pathway (Fig 5e-g, Extended data Fig. 7b), Interestingly, SARS infection had a bit stronger effect on phosphorylation of p65 (Fig. 5f), as a measure of activity via the NF- κ B pathway in infected cells, but the gene transcription of cytokines IL-6 and IL-8 appears not statistically different between SARS and CoV-2 viruses (Fig. 5 g). This is likely due to multiple pathways that can co-regulate gene expression of IL-6 and IL-8, in addition to NF- κ B, such as MAPK and AP-1. However, these results indeed confirm that treatment with inhibitors like GRL-0617, apart from inhibiting the viral replication could assist in mounting an anti-viral immune response in the epithelia, the prime site of pathogen entry and thus inhibiting the pathogenesis at the early stages.

Fig. 5c, d, g: again 50-100 μ M is a very high dose of the protease inhibitor GRL-0617. The authors need to demonstrate that this dose is selectively inactivating PLpro-CoV2 and not host or other viral proteases, the inhibition of which may contribute to the noted cellular efficacy.

We thank the reviewer for this comment. The selectivity of GRL-0617 or other naphthalene-based compounds have been tested with cellular DUBs ([Baez-Santos, et al, J. Med. Chem. 2014](#), [Ratia, et al, PNAS, 2008](#)), and none of the tested DUBs (USP2, USP7, USP8, USP18, USP20, USP21, USP28, UCH-L1, UCH-L3, DEN1, caspase 3 and cathepsin K) was inhibited by naphthalene-based compounds even at 100 μ M.

Lines 330-332: The authors indicate using TALON beads purification with buffers containing 2mM DTT or 1mM TCEP, please confirm that these were the buffer conditions used.

We used the 2 mM DTT for the purification of SARS, MERS, SARS CoV2 PLpro. For crystallization, we tested different buffers. 1 mM TCEP was included in the final buffers for proteins used for crystallization. We also described that we used different pH buffer for crystallization.

Lines 353-368: The description of the protein concentrations used in the crystallization is confusing and should be clarified with more detail. The authors state the concentration of the complex is 250 microM, and indicate a 1:1 protein:mother liquor dilution yet state the protein concentration as still being 250 microM. Are the authors referring to the initial or final concentration? The authors go on to describe optimized crystals using a different protein concentration. Was this different concentration achieved by varying the drop ratios or by diluting the protein? There is no information on murineISG15, the buffer it was in and whether it was modified at the C-term to facilitate a covalent interaction with C111S and whether there was additional gel filtration step after the complex was formed.

We have now corrected protein concentration and included detailed information for the crystallization.

In the crystallographic model of PLproCoV2 in complex with IGS15 the hydroxyl from C111S is 2.3 Å from the C-terminal carbonyl oxygen of murine IGS15, this is too close for what is likely to be an unfavorable interaction.

We thank the reviewer for this specific correction. Based on the suggested catalytic mechanism on PLpro we have now refined the structure for C111S and C-terminus of IGS15. The distance between C111S and C-terminal carbonyl oxygen of mISG15 is 3.8 Å. The new coordinates are submitted to PDB to replace our previous model and statistics for the refinement is updated in Ext. data Table1.

(Baez-Santos, John and Mesecar, Antiviral. Res. 2015)

[redacted]

Lines 401-440: The methods describing the set up for the MD simulation of PLproCoV2 with K48-Ub2 and the MD simulation of PLproCoV2 with mISG15 are different. Since the authors are comparing the results of these two simulations the setup should be as similar as possible, accepting the differences in the starting models.

To generate the starting model of PLproCoV2 with K48-Ub2 for the MD simulation, the authors used coordinates from two different structures, PDB-ID 6W9C for apo-PLproCoV2 and PDB-ID 5E6J for K48-Ub2. However, it is clear from a comparison of crystallographic structures of apo-PLproCoV2 and PLproCoV2 in complex with substrates, as well as structures of other PLpro enzymes, that there is considerable movement around the active site when substrate is bound. Therefore, it seems unsurprising that in the MD simulation using apo-PLproCoV2 as the starting model for a complex with K48-Ub2 superimposed, that the K48-Ub2 moves away from the active site. It would be more appropriate to use the coordinates of PLproCoV2 taken from a substrate complex, or use a homology model based on a substrate complex as the starting model for the MD simulation.

We thank the reviewer for the valid feedback. To address the concern that the initial model of the protease was in an apo state with the K48-Ub₂ substrate added by modelling, we performed additional multi-microsecond simulations based on the new crystal structure. Importantly, all simulations were now run in triplicate. In the setup suggested by the reviewer, we used the substrate-bound PLproCoV2 obtained here as starting structure, with K48-Ub₂ replacing mISG15. In three simulations of 2.7 μs each, we observed one full dissociation of the distal ubiquitin and several transient partial separations. As described in our response to reviewer #1, the full and partial dissociations were associated with water penetrating between Ile44^{Ub} and Thr75^{CoV2}. Therefore, consistently and independent of the initial model, the observed dissociations occur with the same mechanism of water first entering into the hydrophobic contact between Ile44^{Ub} and Thr75^{CoV2}. We included all new simulation data into the manuscript (see Extended data Fig. 2f). In the Methods

section, we make it clear that we used an X-ray structure for the PLproCoV2:mISG15 complex, whereas we used two models for the PLproCoV2:K48-Ub₂ complex.

Reviewer Reports on the First Revision:

Referee #1 (Remarks to the Author):

The authors have significantly improved the previous version of their manuscript. It should now be suitable for publication. A few minor comments remain:

line 52: "...caused a worldwide outbreak of the respiratory disease COVID-19 by the beginning of 2020" - this sounds as if the pandemic is already over.

line 55: The MERS outbreak is still ongoing ...

line 56: Replace "SARS" by "SARS-CoV".

line 84: ... competition was much less effective (delete "a").

line 109: A reference should be provided for the MERS-CoV-PLpro/ISG15 complex, not only the PDB code.

line 196: place "serpins" in parantheses (). Why is it capitalized?

line 366: Replace "protein expression" by "protein production". One can express genes, but not proteins (even though there is even a scientific journal carrying the incorrect term "protein expression" in its title...).

Referee #2 (Remarks to the Author):

The authors have adequately address this reviewers' specific comments with new data and/or clarification. The overarching concern of novelty still remains. As noted originally, "the major findings of this study are largely expected based on the high similarities between SARS-CoV-2 and SARS-CoV." Thus, the groundbreaking work here appears to be mostly based on nuance between the two systems. Given the importance of SARS2, perhaps nuance is sufficient for the highest tier papers, but this would otherwise be unusual for any other viral system in any other setting.

Referee #3 (Remarks to the Author):

This revised manuscript from Shin and co-workers is highly responsive to the reviewers' comments. The additional results provided further strengthen this impressive body of work investigating the substrate specificity of Severe acute respiratory syndrome coronavirus 2 papain-like protease and rigorously comparing it to the activity of Severe acute respiratory syndrome coronavirus papain-like protease.

Minor comments for the authors' consideration:

1. Nomenclature for the enzymes is still confusing. SARS is the name of the disease caused by SARS-CoV. You should include the virus as part of the abbreviation for the enzyme that comes from the virus. It would be more appropriate to use SC-PLpro and SC2-PLpro, or SCoV-PLpro and SCoV2-PLpro. Some people use superscript, while others do not. It is not required to use the superscript designation for the enzyme.

2. Line 56: SARS-CoV-2 shares high sequence identify with SARS-CoV.
3. Line 60-62: add that inhibition of PLpro blocked SARS-CoV replication (ref).
4. Line 228: viral replication of SARS-CoV in Vero E6 cells
5. Line 255: what does lower effect mean? Do you mean to compare the level of ISGylation of IRF-3 at a single time point? The western blot data in 5e and 5f seem to be very similar for the two virus infections. The differences detected in 5g may be due to PLpro or to differences in other IFN antagonist activities (as the authors point out later in this paragraph).
6. Line 261: a reference should be added (type I and type III responses).

Referee #4 (Remarks to the Author):

The authors have addressed most of my questions and concerns, with the exception of the following:

1. Selectivity profiling of GRL-0617: the authors refer to previously published reports in which only 12 proteases were profiled. At the very least the compound should be investigated in commercially-available DUB, protease, and kinase panels.
2. Every panel in Figure 5 uses GRL-0617 and the authors highlight these findings in their abstract: "Pharmacological inhibition of CoV2PLpro blocks the virus-induced cytopathogenic effect, fosters the anti-viral interferon pathway and reduces viral replication in infected cells." However the concentration of GRL-0617 used in panels e, f, and g of Fig. 5 is not indicated in the figure, legend, or methods (that I can find). The compound concentrations should be clearly stated in the legends. Significant effects of GRL-0617 are only evident at 100µM in panels c and d of Fig. 5, and it is highly unlikely that GRL-0617 is only acting on CoV2PLpro in cells at this concentration.

I understand that this is a highly competitive field and that the data in this manuscript should be published in a time-sensitive manner. Given this, extensive profiling of the likely off-target effects of GRL-0617 is not achievable in a suitably timely manner. While some of the cellular effects shown by the authors may be due to GRL-0617-induced CoV2PLpro inhibition, the compound is not well-characterized enough and is too weak to state this conclusion definitively. Therefore it would be in the best interest of all parties involved if the authors clearly acknowledge these uncertainties in the interpretation of their data.

To clarify:

- In lines 176-177, modify the statement to "These results showed that GRL-0617 inhibits both CoV2PLpro and SARSPLpro." (That is, remove "is an effective inhibitor of...")
- At the conclusion of the section ending on line 250, include statements that 1) acknowledge that GRL-0617 is a relatively weak CoV2PLpro inhibitor, ii) the cellular target specificity is incompletely defined, and iii) the concentrations that were used may have affected the function of other cellular proteins.
- The language in the abstract referring to pharmacological inhibition of SARSPLpro affecting virus-induced cytopathogenicity, effects on the anti-viral interferon pathway, and reduction of viral replication in infected cells should be re-worded with more caution given that these studies were exclusively performed with GRL-0617, a relatively weak and incompletely characterized inhibitor.

Author Rebuttals to First Revision:

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The authors have significantly improved the previous version of their manuscript. It should now be suitable for publication. A few minor comments remain:

line 52: "...caused a worldwide outbreak of the respiratory disease COVID-19 by the beginning of 2020" - this sounds as if the pandemic is already over.

We thank reviewer for this specific comment. We have corrected the sentence.

line 55: The MERS outbreak is still ongoing ...

We thank reviewer for this specific comment. We have removed MERS as we are focusing more on SARS and SARS-CoV-2.

line 56: Replace "SARS" by "SARS-CoV".

We thank reviewer for this specific comment. We have changed SARS to SARS-CoV.

line 84: ... competition was much less effective (delete "a").

We thank reviewer for this detailed correction and we have deleted "a".

line 109: A reference should be provided for the MERS-CoV-PLpro/ISG15 complex, not only the PDB code.

We thank reviewer for the comment and now the reference for the structure is included.

line 196: place "serpins" in parantheses (). Why is it capitalized?

We thank reviewer for this specific comment. We have now placed serpins in parantheses. Since SEPRIN B3 is used for referring protein, we left SERPIN B3 as capital for naming SEPRIN B3 protein.

line 366: Replace "protein expression" by "protein production". One can express genes, but not proteins (even though there is even a scientific journal carrying the incorrect term "protein expression" in its title...).

We thank reviewer for this specific comment, the protein expression is now changed to protein production.

Referee #2 (Remarks to the Author):

The authors have adequately address this reviewers' specific comments with new data and/or clarification. The overarching concern of novelty still remains. As noted originally, "the major findings of this study are largely expected based on the high similarities between SARS-CoV-2 and SARS-CoV." Thus, the groundbreaking work here appears to be mostly based on nuance between the two systems. Given the importance of SARS2, perhaps nuance is sufficient for the highest tier papers, but this would otherwise be unusual for any other viral system in any other setting.

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Minor comments for the authors' consideration:

1. Nomenclature for the enzymes is still confusing. SARS is the name of the disease caused by SARS-CoV. You should include the virus as part of the abbreviation for the enzyme that comes from the virus. It would be more appropriate to use SC-PLpro and SC2-PLpro, or SCoV-PLpro and SCoV2-PLpro. Some people use superscript, while others do not. It is not required to use the superscript designation for the enzyme.

We thank reviewer for this specific comment, we have discussed the nomenclature with editor and changed the name to SCoV-PLpro and SCoV2-PLpro.

2. Line 56: SARS-CoV-2 shares high sequence identify with SARS-CoV.

We thank reviewer for this specific comment. We have corrected the sentence.

3. Line 60-62: add that inhibition of PLpro blocked SARS-CoV replication (ref).

We thank reviewer for this specific comment. We have corrected the sentence.

4. Line 228: viral replication of SARS-CoV in Vero E6 cells

We thank reviewer for this specific comment. We have corrected the sentence and moved the sentence to the beginning of the paragraph.

5. Line 255: what does lower effect mean? Do you mean to compare the level of ISGylation of IRF-3 at a single time point? The western blot data in 5e and 5f seem to be very similar for the two virus infections. The differences detected in 5g may be due to PLpro or to differences in other IFN antagonist activities (as the authors point out later in this paragraph).

We thank reviewer for this critical comment. We clarified this point by indicating: SARS-CoV infection had a consistently lower effect of GRL-0617 on restoring the ISGylation and phosphorylation level of IRF3, and on the expression of IFN responsive genes as compared to SARS-CoV-2

6. Line 261: a reference should be added (type I and type III responses).

We thank reviewer for this specific comment. We have included the reference.

Referee #4 (Remarks to the Author):

The authors have addressed most of my questions and concerns, with the exception of the following:

1. Selectivity profiling of GRL-0617: the authors refer to previously published reports in which only 12 proteases were profiled. At the very least the compound should be investigated in commercially-available DUB, protease, and kinase panels.

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I understand that this is a highly competitive field and that the data in this manuscript should be published in a time-sensitive manner. Given this, extensive profiling of the likely off-target effects of GRL-0617 is not achievable in a suitably timely manner. While some of the cellular effects shown by the authors may be due to GRL-0617-induced CoV2PLpro inhibition, the compound is not well-characterized enough and is too weak to state this conclusion definitively. Therefore it would be in the best interest of all parties involved if the authors clearly acknowledge these uncertainties in the interpretation of their data.

We thank reviewer for raising these concerns and suggestion. We appreciate also a critical view on potential additional effects of GRL-0617. We have re-worded the conclusion to adequately present our data and have included the concentration of GRL-0617 for Fig. 5 e-g in figure legends. However, additional studies (DOI: <https://doi.org/10.1101/2020.06.18.160614>) using inhibitors of SCoV-PLpro indicated similar effects on viral replication and release. Thus, we feel that together both studies support an important role of inhibition of PLpro on viral spread.

To clarify:

- In lines 176-177, modify the statement to “These results showed that GRL-0617 inhibits both CoV2PLpro and SARSPLpro.” (That is, remove “is an effective inhibitor of...”)

We thank reviewer for this suggestion and we have removed the word for “effective inhibitor”.

- At the conclusion of the section ending on line 250, include statements that 1) acknowledge that GRL-0617 is a relatively weak CoV2PLpro inhibitor, ii) the cellular target specificity is incompletely defined, and iii) the concentrations that were used may have affected the function of other cellular proteins.

We thank reviewer for this suggestion and we have included below statements in the manuscript.

“Considering that GRL-0617 is a relatively weak inhibitor used at high concentrations that may lead to off-target effects, further studies for developing potent and selective inhibitor for PLpro are needed.”

- The language in the abstract referring to pharmacological inhibition of SARSPLpro affecting virus-induced cytopathogenicity, effects on the anti-viral interferon pathway, and reduction of viral replication in infected cells should be re-worded with more caution given that these studies were exclusively performed with GRL-0617, a relatively weak and incompletely characterized inhibitor.

We thank reviewer for this specific comment. We have corrected the sentence as below.

“Importantly, inhibition of SCoV2-PLpro with GRL-0617 impairs...”