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Peer Review File

A divergent betacoronavirus with a functional furin cleavage site in South American bats

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The study presented by Takada et al. described the detection and whole genome characterization of a novel bat CoV associated with Brazilian Pterotonus parnellii bats that was previously reported in the same bat species from Costa Rica. This CoV possibly constitutes a novel subgenus within the Betacoronavirus genus and harbors a functional furin cleavage site.
Despite the addition of a “limitation” section presenting the lack of several experiments (including virus-receptor interaction studies), the authors claim that their study provides important insights of the zoonotic potential of this novel CoV (abstract section l.58, and discussion l.256).
Apart from this overstatement that should be minimized, some changes have to be made to improve the manuscript:
- Results section:
o Add a phylogeny based on partial RdRP region to include the Costa Rican CoV sequences
o Include the ICTV demarcation criteria that allow the authors to conclude that the CoV constitutes a novel subgenus within the Betacoronavirus genus
o Provide as supplementary data the whole Spike amino-acid alignment to confirm the upstream position of the furin cleavage site of the novel CoV compared to other betacoronaviruses. The partial alignment provided in Figure 4B is not sufficient
- Material & methods section:
o The protocol describing how the whole genome of the novel CoV was obtained is missing
o Add ethical considerations and authorizations for bat sampling
o Labelling of figures 2E and S3 do not correspond to pairwise distance comparison mentioned in lines 347-348
o Parameters for CD-HIT clustering are missing (l.359 and l.374)

Reviewer #2

(Remarks to the Author)
In this study, Takada and colleagues identify a new bat derived beta-CoV from South American bats with a functional FCS. The authors demonstrate that the FCS is cleaved during protein production. The study is intriguing but needs additional data to be scientifically rigorous and to support all the claims.

1. Authors suggest that that the FCS region could be a hotspot for cleavage site incorporation. The authors should consider doing a recombination analysis across reported bat derived beta-CoV sequences to assess whether this region could indeed recombine between bat derived coronaviruses.

2. What happened to Orf 7? Was it not identified at the 5’ end of Orf 8b? Were other accessory genes not identified or are the authors suggesting that the virus only has once accessory gene?

3. AlphaFold3 structure confidence is low, which is expected since the spike of the new virus is divergent from what is reported in the database. To strengthen the structural prediction, I would recommend validating and reconstructing the structure using another model.

4. In figure 3D, WT BRZ S full length band is missing. It is unlikely that the entire spike is being cleaved with such high efficiency. Try loading different concentrations of the protein. Or is the protein being fully cleaved during production in Expi293 F cells, which is also unexpected. Similarly, for GSAS mutant protein, a light band is observed for full length S. Please load equal amounts for all proteins to be able to compare them side-by-side. With such low levels of full-length S for GSAS mutant, it is hard to assess whether any of it is being cleaved. I would also suggest using protease inhibitors, like furin inhibitors to demonstrate that the RDAR site is indeed cleaved by proteases in the cells. The authors claim that RDAR is a furin cleavage site, which needs to be demonstrated by specifically blocking the action of furin in the cells and demonstrating the subsequent loss of S cleavage. Another aspect that will further strengthen the study is to demonstrate whether the FCS is functional in human lung and intestinal cells or organoids (adds relevance for zoonotic potential), along with bat cells derived from the bat species where the virus sequence was detected.

5. In figure 3F, GSAS/PP Spike produces a string band just above the 35KDa marker. What does the band correspond to? Is there a secondary cut site (like S2' site) which now becomes more accessible for cleavage?

6. For figure 4, the authors report that 9/15 beta-CoVs possess FCSs. These are alignment-based predictions only so I would suggest taming down the claims and calling them putative FCSs. Alternatively, authors could synthesize these sequences and demonstrate experimentally that they are indeed functional S1/S2 cleavage sites.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed all the concerns I had.

Just one point for clarity: please highlight the position of the FCS to help reader find it in the alignment presented in Supp. figure 3, along with some additional annotations (S1, S2, NTD, RBD...)

Reviewer #2

(Remarks to the Author)
Thank you for revising the manuscript, but additional experiments and clarifications are required to address the furin data and for scientific rigour.

1. In figure 3E, siRNA against furin leads to only partial knockdown, but in figure 3F, siRNA against furin leads to a complete furin knockdown in the cells. Why is there this discrepancy?

2. If during siRNA is being used to knockdown furin in these cells, what measures are being taken to reintroduce furin expression in these cells? How is the existing siRNA not inhibiting furin ectopic expression?

3, Figures 3E and 3F currently are contradictory and further confuses the results and overall conclusion of this study. Please include full immunoblots for all the experiments, at least in the supplementary.

4. Figure R2B is not conclusive and further optimizations are required for furin and spike expression. In the revised data, the knockdown of endogenous furin is confusing (and not reproducible between figures 3E and 3F) and not complete, so the role of bat furin in the likely presence of endogenous human furin is inconclusive.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have now addressed my concerns.

RE: NCOMMS-25-92348-T

Title: A divergent betacoronavirus with a functional furin cleavage site in South American bats

Reviewer #1 (Remarks to the Author):

The study presented by Takada et al. described the detection and whole genome characterization of a novel bat CoV associated with Brazilian Pterotonus parnellii bats that was previously reported in the same bat species from Costa Rica. This CoV possibly constitutes a novel subgenus within the Betacoronavirus genus and harbors a functional furin cleavage site.

Despite the addition of a "limitation" section presenting the lack of several experiments (including virus-receptor interaction studies), the authors claim that their study provides important insights of the zoonotic potential of this novel CoV (abstract section l.58, and discussion l.256).

Apart from this overstatement that should be minimized, some changes have to be made to improve the manuscript:

Response: As requested, we have revised the text to avoid overinterpretation of zoonotic implications. We now frame our conclusions in terms of evolutionary context and processes, rather than directly inferring zoonotic risk or potential (Page 3, lines 58–59; and page 11, lines 253–254).

- Results section:

Add a phylogeny based on partial RdRP region to include the Costa Rican CoV sequences

Response: We included a phylogeny based on the partial RdRp (nsp12) region that incorporates the Costa Rican coronavirus sequences in the original manuscript (Fig. 1C). We have clarified this analysis in the revised manuscript (Page 6, line 134).

Include the ICTV demarcation criteria that allow the authors to conclude that the CoV constitutes a novel subgenus within the Betacoronavirus genus

Response: The ICTV-recommended *Coronavirinae* marker gene set (nsp5, nsp12, nsp13, nsp14, nsp15, and nsp16) has already been analysed and is presented in Fig. 1E in the original manuscript. We have clarified this point in the revised manuscript and explicitly link these analyses to the ICTV subgenus demarcation criteria (Page 6, lines 137–138).

Provide as supplementary data the whole Spike amino-acid alignment to confirm the

upstream position of the furin cleavage site of the novel CoV compared to other betacoronaviruses. The partial alignment provided in Figure 4B is not sufficient

Response: We now provide the complete Spike amino acid alignment as Supplementary Fig. S3. This confirms the upstream position of the furin cleavage site in the novel coronavirus relative to other betacoronaviruses. The partial alignment shown in Fig. 4B has been retained for clarity (Page 32, lines 775–777).

- Material & methods section:
The protocol describing how the whole genome of the novel CoV was obtained is missing

Response: We have now clarified that *de novo* assembly using SPAdes yielded a single coronavirus contig spanning the full-length genome, including the putative 5′ and 3′ UTRs, and have added read-mapping–based validation of the assembly (Page 15, lines 342–348).

Add ethical considerations and authorizations for bat sampling

Response: We have added a description of the ethical considerations and relevant permits and approvals for bat sampling to the Methods section (Page 14, lines 306–317).

Labelling of figures 2E and S3 do not correspond to pairwise distance comparison mentioned in lines 347-348

Response: We apologize for the error in the figure citation. The pairwise distance comparison referred to in lines 347–348 in fact corresponds to Fig. S1, not Fig. S3. The labelling and text have been corrected (Page 16, line 367).

Parameters for CD-HIT clustering are missing (l.359 and l.374)

Response: We now provide the CD-HIT-EST clustering parameters in the Methods section, indicating that identical sequences were removed using CD-HIT-EST v4.8.1 with a 100% sequence identity threshold (-c 1) (Page 16, lines 378–379).

Reviewer #2 (Remarks to the Author):

In this study, Takada and colleagues identify a new bat derived beta-CoV from South American bats with a functional FCS. The authors demonstrate that the FCS is cleaved during protein production. The study is intriguing but needs additional data to be scientifically rigorous and to support all the claims.

1. Authors suggest that that the FCS region could be a hotspot for cleavage site incorporation.

The authors should consider doing a recombination analysis across reported bat derived beta-CoV sequences to assess whether this region could indeed recombine between bat derived coronaviruses.

Response: We thank the reviewer for this suggestion. We agree that demonstrating recombination events would require dedicated analyses across a broad and representative set of bat-derived betacoronavirus sequences. However, given the currently limited availability and uneven sampling of bat-associated betacoronavirus genomes from South America, as well as the scarcity of close relatives of the divergent coronavirus identified in this study, such analyses would be difficult to interpret robustly within the scope of the present study. We have therefore revised the Discussion to acknowledge this limitation and to clarify that distinguishing between the independent acquisition and recombination-driven incorporation of FCSs will require analyses across a more diverse array of bat-associated betacoronaviruses, including currently unsampled lineages, which represents an important direction for future studies (Pages 12–13, lines 284–288).

2. What happened to Orf 7? Was it not identified at the 5' end of Orf 8b? Were other accessory genes not identified or are the authors suggesting that the virus only has once accessory gene?

Response: Accessory gene content varies substantially among betacoronaviruses. Unlike SARS-CoV-2, which encodes ORF7a/7b, MERS-CoV lacks an ORF7 equivalent, or any clear homologous gene, in this genomic region, as reported in previous studies (Fang et al., 2021; Forni et al., 2017). We detected no convincing homology to ORF7 in the novel CoV, including at the 5′ end of ORF8b, despite extensive similarity searches. Therefore, we do not interpret this as a mis-annotation, but rather as reflecting genuine lineage-specific differences in accessory gene composition, which are well documented among betacoronaviruses.

References

1. Fang P, Fang L, Zhang H, Xia S, Xiao S. Functions of Coronavirus Accessory Proteins: Overview of the State of the Art. Viruses. 2021 Jun 13;13(6):1139. doi: 10.3390/v13061139. PMID: 34199223; PMCID: PMC8231932.
2. Forni D, Cagliani R, Clerici M, Sironi M. Molecular Evolution of Human Coronavirus Genomes. Trends Microbiol. 2017 Jan;25(1):35-48. doi: 10.1016/j.tim.2016.09.001. Epub 2016 Oct 19. PMID: 27743750; PMCID: PMC7111218.

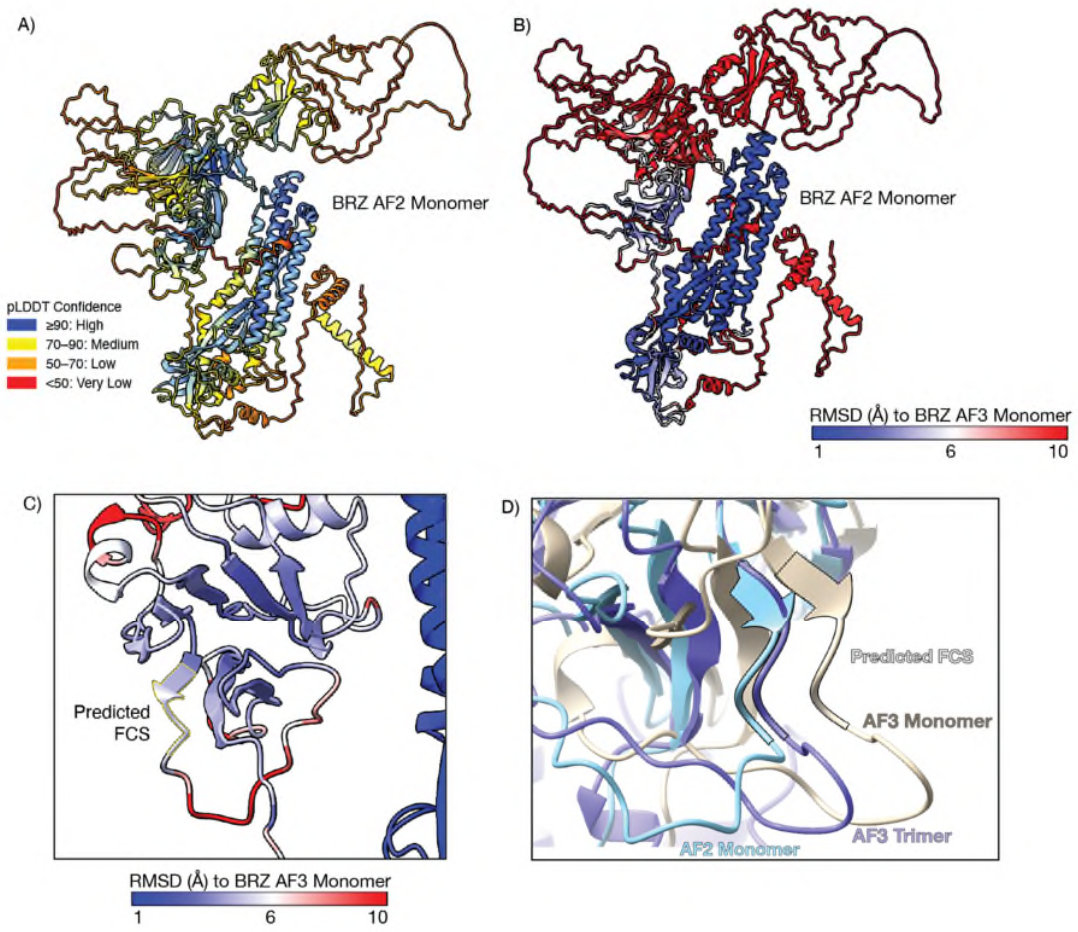
3. AlphaFold3 structure confidence is low, which is expected since the spike of the new virus is divergent from what is reported in the database. To strengthen the structural prediction, I would recommend validating and reconstructing the structure using another model.

Response: We agree with the reviewer that there is a need for further protein structural validation given the divergence of the spike protein. To address this, we generated additional protein structural predictions using alternative models based on different underlying principles to AlphaFold3 (AF3), namely AlphaFold2 (AF2), which does not use a diffusion-based approach, and Boltz-2, an alternative non-DeepMind diffusion-based framework.

Interestingly, the top-ranked BRZ spike monomer predicted by AF2 showed higher overall confidence than the AF3 monomer, with a higher average pLDDT across Cα (61 vs 58) and a higher pTM score (0.57 vs 0.45) (Fig. R1A). Although the Boltz-2 model had an average pLDDT comparable to the other models (60.44), its structural alignment to the experimental HKU5 spike model (PDB 9EH8) using USAlign was poor (TM-score = 0.37) compared to the AF2 and AF3 predicted models (TM-score: AF2= 0.79, AF3 monomer = 0.68, AF3 trimer = 0.83). Consequently, we excluded the Boltz-2 model from further analyses.

The AF2 and AF3 monomer models align well across the majority of subunit 2 and, to a lesser extent, the S1/S2 junction, whereas the N-terminal and receptor-binding domains showed poorer agreement (Fig. R1B). Focusing on the 100 residues flanking the furin cleavage site (FCS), the two models have similar confidence scores (pLDDT: AF2 = 0.67, AF3 = 0.73). When aligned to the AF3 BRZ spike trimer using Matchmaker, the FCS region in the AF2 monomer model showed a deviation of 1.89 Å. The AF2 monomer model was consistent with the AF3 monomer model in which the FCS is predicted as an exposed loop with a short β-sheet, which when aligned deviated by 3.87 Å (Fig. R1C and R1D).

Since the AF2 spike monomer represents a potential improvement over the AF3 model (based on confidence metrics and structural alignment scores) to the experimental structure of HKU5 we now provide this alongside the AF3 structure in the Figshare repository (https://doi.org/10.6084/m9.figshare.31834315). However, as the AF2 and AF3 models are largely consistent in the region surrounding the FCS, which is the focus of our study, we have retained the AF3 model as the primary structural model referenced throughout the manuscript (Page 9, lines 188–193 and Page 18, lines 410–420).



Response Figure R1. (A) AlphaFold2 (AF2)-predicted structure of the BRZ batCoV spike glycoprotein monomer (hereafter referred to as the AF2 structure), colored by pLDDT confidence score as indicated in the key. (B) AF2 structure colored by RMSD relative to the AlphaFold3 (AF3)-predicted BRZ batCoV spike glycoprotein monomer (hereafter referred to as the AF3 structure), as determined using MatchMaker in ChimeraX. (C) Same as in panel B, but focused on the region surrounding the predicted furin cleavage site (FCS; residues 828–831). (D) Superposition of the AF2 and AF3 monomer models onto the BRZ batCoV AF3 trimer model, focusing on the region surrounding the FCS.

4. In figure 3D, WT BRZ S full length band is missing. It is unlikely that the entire spike is being cleaved with such high efficiency. Try loading different concentrations of the protein. Or is the protein being fully cleaved during production in Expi293 F cells, which is also unexpected. Similarly, for GSAS mutant protein, a light band is observed for full length S. Please load equal amounts for all proteins to be able to compare them side-by-side. With such low levels of full-length S for GSAS mutant, it is hard to assess whether any of it is being cleaved.

Response: Since the efficiency of secretion of BRZ batCoV full-length S is very low, we instead examined the expression patterns of full-length and cleaved S proteins in cell lysates following

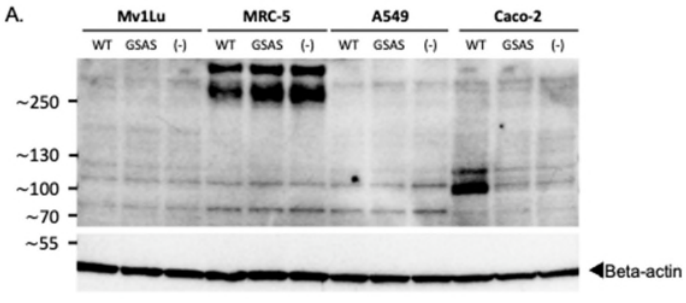
transfection with plasmids encoding BRZ batCoV WT S and the GSAS mutant. While cleavage was clearly observed in the WT S protein, substitution of the putative cleavage site (RDAR) with GSAG completely abolished cleavage, demonstrating that RDAR is a functional furin cleavage site in the BRZ batCoV S protein. We have included these results in the revised manuscript (Figure 3D).

I would also suggest using protease inhibitors, like furin inhibitors to demonstrate that the RDAR site is indeed cleaved by proteases in the cells. The authors claim that RDAR is a furin cleavage site, which needs to be demonstrated by specifically blocking the action of furin in the cells and demonstrating the subsequent loss of S cleavage.

Response: We agree with the reviewer's comment. To confirm that RDAR is specifically cleaved by furin, rather than other proteases, we silenced the furin gene in HEK293T cells by using siRNA. Knockdown (KD) of furin markedly reduced cleavage of the BRZ batCoV S protein. In contrast, re-expression of human furin in furin-KD HEK293T cells restored S cleavage. These results provide strong evidence that proteolytic processing of BRZ batCoV S is dependent on furin. We have added these results in the revised manuscript (Figures 3E and 3F).

Another aspect that will further strengthen the study is to demonstrate whether the FCS is functional in human lung and intestinal cells or organoids (adds relevance for zoonotic potential), along with bat cells derived from the bat species where the virus sequence was detected.

Response: To examine whether the FCS of BRZ batCoV-S is functional in human lung and intestinal cells, we expressed BRZ batCoV S protein in human intestinal and lung-derived cells. However, S protein expression was below the detection limit in these cells (Response Figure R2A), likely due to low transfection efficiency and/or reduced protein expression levels under the conditions used. Since a cell line of *Pteronotus parnellii*, from which the virus sequence was obtained, is not available, we tested several bat kidney-derived cell lines; however, S protein expression was not reliably detected in these systems (Response Figure R2B). To further address this point, we performed a complementation experiment using furin derived from a bat of the genus *Pteronotus* in furin-depleted HEK293T cells. Notably, re-expression of bat furin restored S cleavage, comparable to human furin, as shown in Figure 3F. These results demonstrate that the FCS of BRZ batCoV S protein can be functionally processed by both human and bat furin.



Response Figure 2. Expression of BRZ batCoV-S in (A) lung and intestinal cell lines and (B) bat kidney-derived cell lines. Cells were transfected with the BRZ batCoV-S expression plasmid, and cell lysates were collected at 48 h post-transfection and analyzed by western blotting using anti-BRZ batCoV-S mouse serum. Spike expression was below the detection limit in these cell lines, and no clear bands corresponding to full-length or cleaved S were observed.
Mv1Lu; mink (M. vison) lung cells, MRC-5 and A549; human lung cells, Caco-2; human intestinal cells, ZFBK11-97; *E. gambianus* kidney cells, ZFBK13-76E; *E. helvum* kidney cells, ZFBK15-137RA; *R. aegyptiacus* kidney cells, SuBK12-08; *M. schreibersii* kidney cells.

5. In figure 3F, GSAS/PP Spike produces a string band just above the 35KDa marker. What does the band correspond to? Is there a secondary cut site (like S2' site) which now becomes more accessible for cleavage?

Response: Based on our analysis, this band most likely represents a non-specific degradation product of GSAS/PP Spike rather than cleavage at a defined secondary site such as S2′. This interpretation is supported by the absence of this band in both wild-type and GSAS Spike under identical experimental conditions. To avoid potential confusion and maintain clarity of the main conclusion, we have excluded the GSAS/PP Spike data from the revised manuscript and instead focused on the GSAS mutant, which directly addresses the role of the RDAR cleavage site.

6. For figure 4, the authors report that 9/15 beta-CoVs possess FCSs. These are alignment-based predictions only so I would suggest taming down the claims and calling them putative FCSs. Alternatively, authors could synthesize these sequences and demonstrate experimentally that they are indeed functional S1/S2 cleavage sites.

Response: We agree that the identification of these motifs is based on sequence alignment and motif analysis and hence does not demonstrate functional S1/S2 cleavage *per se*. Accordingly, we have revised the manuscript to consistently refer to these sites as putative FCSs and have toned down the corresponding statements to avoid overinterpretation (Page 10, line 230 and

233). While we agree that experimental validation of the cleavage activity of these sequences would indeed be of interest, this is beyond the scope of the present study.

RE: NCOMMS-25-92348A

Title: A divergent betacoronavirus with a functional furin cleavage site in South American bats

Reviewer #1 (Remarks to the Author):

The authors have addressed all the concerns I had.

Just one point for clarity: please highlight the position of the FCS to help reader find it in the alignment presented in Supp. figure 3, along with some additional annotations (S1, S2, NTD, RBD...)

Response: We thank the reviewer for this suggestion. We now highlight the position of the FCS in Supplementary Fig. S6. We also considered adding broader S1, S2, NTD, and RBD annotations; however, because the viruses included in this alignment are highly divergent, assigning precise domain boundaries across all sequences based on the alignment alone would be unreliable and potentially misleading. We have therefore focused the annotation on the key feature relevant to this figure, namely the putative FCS/S1–S2 cleavage region, and have clarified this in the figure legend (Supplementary Fig. S6).

Reviewer #2 (Remarks to the Author):

Thank you for revising the manuscript, but additional experiments and clarifications are required to address the furin data and for scientific rigour.

1. In figure 3E, siRNA against furin leads to only partial knockdown, but in figure 3F, siRNA against furin leads to a complete furin knockdown in the cells. Why is there this discrepancy?

Response: We thank the reviewer for pointing this out. To directly address this concern, we re-ran the samples from Figures 3E and 3F together on the same gel and blot (Supplementary Fig. S3). This experiment confirmed that furin knockdown by siRNA was consistently partial across both experimental conditions. The apparent discrepancy between the original Figures 3E and 3F was due to differences in exposure settings. In Fig. 3F, the samples were run with the furin re-expression conditions, which contain substantially higher levels of furin. Consequently, the exposure was optimized to avoid saturation of the re-expression lanes, making the residual endogenous furin signal difficult to detect. To illustrate this point, we now provide images of

the same blot acquired at both short and long exposure times (Supplementary Fig. S3), demonstrating that the endogenous furin signal remains detectable in the siRNA-treated cells at longer exposure. We have also clarified this point in the Fig. 3F legend.

2. If during siRNA is being used to knockdown furin in these cells, what measures are being taken to reintroduce furin expression in these cells? How is the existing siRNA not inhibiting furin ectopic expression?

Response: We thank the reviewer for this important comment. To restore furin expression in furin-depleted cells, we used a codon-optimized synthetic furin expression construct. Codon optimization alters the nucleotide sequence while preserving the amino acid sequence of the encoded protein and is therefore expected to substantially reduce the susceptibility of the ectopically expressed furin transcript to siRNA-mediated knockdown targeting the endogenous sequence. This approach is widely used in rescue experiments of this type to decouple ectopic expression from siRNA-mediated suppression. We have added a clear explanation of this to the Methods section (Pages 19-20, lines 454-457).

3, Figures 3E and 3F currently are contradictory and further confuses the results and overall conclusion of this study. Please include full immunoblots for all the experiments, at least in the supplementary.

Response: We thank the reviewer for this important comment. As described in our response to Comment 1, we re-ran the samples from Figures 3E and 3F on the same gel and blot, confirming that furin knockdown was consistently partial across both experimental conditions and resolving the apparent discrepancy between the two figures (Supplementary Fig. S3). In addition, we now provide the full, uncropped immunoblot images for all relevant western blot experiments in Supplementary Figures S4 and S5, as requested.

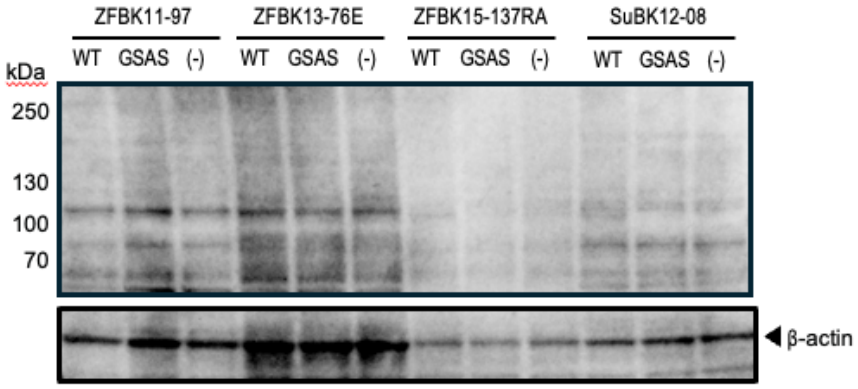
4. Figure R2B is not conclusive and further optimizations are required for furin and spike expression. In the revised data, the knockdown of endogenous furin is confusing (and not reproducible between figures 3E and 3F) and not complete, so the role of bat furin in the likely presence of endogenous human furin is inconclusive.

Response: We thank the reviewer for this important comment. As described in our responses to Comments 1 and 3, we re-ran the relevant samples on the same gel and blot and have provided the full, uncropped immunoblots, which confirm that furin knockdown is partial rather than complete and explains the apparent discrepancy between Figures 3E and 3F (Supplementary Figures S3, S4, and S5).

We agree that the furin knockdown/re-expression experiment should be interpreted with

caution. Accordingly, we have revised the text (Pages 9–10, lines 210–213) to avoid overinterpretation of these data. Rather than claiming complete replacement of endogenous human furin, we now describe the results as supporting the ability of bat furin to promote spike processing under conditions in which endogenous furin is reduced but not eliminated. We also clarify that residual endogenous human furin may contribute to spike cleavage in this assay.

Regarding “Response Figure 2B” in our previous response letter, we acknowledge that spike expression in bat cell lines is technically challenging. Beyond transfection, we attempted to express BRZbatCoV spike protein by transducing bat cell lines with a lentiviral vector. However, spike expression remained below the detection limit despite additional optimization efforts. We have included this information in “Response Figure 1” below to clarify that further optimization was attempted, but did not result in detectable spike expression.



Response Figure 1. Attempted expression of BRZ batCoV spike protein in bat kidney-derived cell lines.

Bat kidney-derived cell lines were transduced with lentiviral vectors encoding BRZ batCoV spike protein (WT), the GSAS mutant (GSAS), or empty vector (−). Cell lysates were collected 48 h post-transduction and analyzed by western blotting using mouse anti-BRZ batCoV spike serum. No distinct bands corresponding to full-length or cleaved spike protein were detected in any of the cell lines tested.

ZFBK11-97, E. gambianus kidney cells; ZFBK13-76E, E. helvum kidney cells; ZFBK15-137RA, R. aegyptiacus kidney cells; SuBK12-08, M. schreibersii kidney cells