nature research

Peer Review Information

Journal: Nature Microbiology
Manuscript Title: **The furin cleavage site in the SARS-CoV-2 spike protein is required for transmission in ferrets**
Corresponding author name(s): Wendy Barclay

Reviewer Comments & Decisions:

Decision Letter, initial version:

Dear Dr. Barclay,

Thank you for your patience while your manuscript "The furin cleavage site of SARS-CoV-2 spike protein is a key determinant for transmission due to enhanced replication in airway cells" was under peer-review at Nature Microbiology. It has now been seen by 4 referees, whose expertise and comments you will find at the of this email. You will see from their comments below that while they find your work of interest, some important points are raised. We are very interested in the possibility of publishing your study in Nature Microbiology, but would like to consider your response to these concerns in the form of a revised manuscript before we make a final decision on publication.

In particular, you will see that referee #1 has concerns over the lack of appropriate referencing in the Introduction section. This referee also feels that "The data on the use of 'furin inhibitor' is not convincing", and states that "it may be best to eliminate these data if more convincing inhibition cannot be obtained (by Western blot)." Please note that editorially, we feel it will be important to address these concerns experimentally. Furthermore, referee #1 is not convinced by the data on IFITM and amphotericin B, and editorially, we would favour to have these concerns addressed experimentally. Referee #2 suggests to more carefully cite previous studies, and asks to provide a more detailed discussion of "why deletion of the furin cleavage site reduces Calu-3/HAE entry although it is dispensable for cathepsin L usage and high levels of cathepsin L are available for S protein activation in these cells". Referee #3 suggests to extend the Discussion. Referee #4 feels that "it would be important to demonstrate that similar results are obtained using differentiated Caco-2 and Calu-3 cells, which more closely model in vivo, and using at least n = 3 HAE donors." Editorially, we share referee 4's view that this would be an important experiment. This referee also asks to comment on whether there was increased cell death in the SARS cells, and to discuss the results in Figure 3D. Referee #4 also mentions that information is missing on how the experiment showing that IFITM3 impacts viral entry was performed, including which controls were used.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or

unlikely to yield a meaningful outcome.

If you have not done so already please begin to revise your manuscript so that it conforms to our Article format instructions at http://www.nature.com/nmicrobiol/info/final-submission/

The usual length limit for a Nature Microbiology Article is six display items (figures or tables) and 3,000 words. We have some flexibility, and can allow a revised manuscript at 3,500 words, but please consider this a firm upper limit. There is a trade-off of ~250 words per display item, so if you need more space, you could move a Figure or Table to Supplementary Information.

Some reduction could be achieved by focusing any introductory material and moving it to the start of your opening 'bold' paragraph, whose function is to outline the background to your work, describe in a sentence your new observations, and explain your main conclusions. The discussion should also be limited. Methods should be described in a separate section following the discussion, we do not place a word limit on Methods.

Nature Microbiology titles should give a sense of the main new findings of a manuscript, and should not contain punctuation. Please keep in mind that we strongly discourage active verbs in titles, and that they should ideally fit within 90 characters each (including spaces).

We strongly support public availability of data. Please place the data used in your paper into a public data repository, if one exists, or alternatively, present the data as Source Data or Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. For some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories can be found at https://www.nature.com/nature-research/editorial-policies/reporting-standards#availability-of-data.

Please include a data availability statement as a separate section after Methods but before references, under the heading "Data Availability". This section should inform readers about the availability of the data used to support the conclusions of your study. This information includes accession codes to public repositories (data banks for protein, DNA or RNA sequences, microarray, proteomics data etc…), references to source data published alongside the paper, unique identifiers such as URLs to data repository entries, or data set DOIs, and any other statement about data availability. At a minimum, you should include the following statement: "The data that support the findings of this study are available from the corresponding author upon request", mentioning any restrictions on availability. If DOIs are provided, we also strongly encourage including these in the Reference list (authors, title, publisher (repository name), identifier, year). For more guidance on how to write this section please see:
http://www.nature.com/authors/policies/data/data-availability-statements-data-citations.pdf

To improve the accessibility of your paper to readers from other research areas, please pay particular attention to the wording of the paper's opening bold paragraph, which serves both as an introduction and as a brief, non-technical summary in about 150 words. If, however, you require one or two extra sentences to explain your work clearly, please include them even if the paragraph is over-length as a result. The opening paragraph should not contain references. Because scientists from other sub-disciplines will be interested in your results and their implications, it is important to explain essential but specialised terms concisely. We suggest you show your summary paragraph to colleagues in other

fields to uncover any problematic concepts.

If your paper is accepted for publication, we will edit your display items electronically so they conform to our house style and will reproduce clearly in print. If necessary, we will re-size figures to fit single or double column width. If your figures contain several parts, the parts should form a neat rectangle when assembled. Choosing the right electronic format at this stage will speed up the processing of your paper and give the best possible results in print. We would like the figures to be supplied as vector files - EPS, PDF, AI or postscript (PS) file formats (not raster or bitmap files), preferably generated with vector-graphics software (Adobe Illustrator for example). Please try to ensure that all figures are non-flattened and fully editable. All images should be at least 300 dpi resolution (when figures are scaled to approximately the size that they are to be printed at) and in RGB colour format. Please do not submit Jpeg or flattened TIFF files. Please see also 'Guidelines for Electronic Submission of Figures' at the end of this letter for further detail.

Figure legends must provide a brief description of the figure and the symbols used, within 350 words, including definitions of any error bars employed in the figures.

When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-research/editorial-policies/image-integrity">Digital Image Integrity Guidelines. and to the following points below:

-- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
-- that control panels for gels and western blots are appropriately described as loading on sample processing controls
-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

Please include a statement before the acknowledgements naming the author to whom correspondence and requests for materials should be addressed.

Finally, we require authors to include a statement of their individual contributions to the paper -- such as experimental work, project planning, data analysis, etc. -- immediately after the acknowledgements. The statement should be short, and refer to authors by their initials. For details please see the Authorship section of our joint Editorial policies at http://www.nature.com/authors/editorial_policies/authorship.html

When revising your paper:

* include a point-by-point response to any editorial suggestions and to our referees. Please include your response to the editorial suggestions in your cover letter, and please upload your response to the referees as a separate document.

* ensure it complies with our format requirements for Letters as set out in our guide to authors at www.nature.com/nmicrobiol/info/gta/

* state in a cover note the length of the text, methods and legends; the number of references; number and estimated final size of figures and tables

* resubmit electronically if possible using the link below to access your home page:

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Please ensure that all correspondence is marked with your Nature Microbiology reference number in the subject line.

Nature Microbiology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. This applies to primary research papers only. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

We hope to receive your revised paper within three weeks. If you cannot send it within this time, please let us know.

We look forward to hearing from you soon.

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Reviewer Expertise:

Referee #1: Coronavirus and viral entry
Referee #2: Coronavirus, TMPRSSR2, furin-sites and IFITM
Referee #3: Deep sequencing of viruses
Referee #4: Animal model

Reviewers Comments:

Reviewer #1 (Remarks to the Author):

This is a timely and important study, in what is currently an active area of SARS2 research – the function of the S1/S2 cleavage site. The work is well done

Some of the referencing in the Introduction is incomplete or inaccurate – Holshue (line 36) does not directly address the statement concerning WHI, and Belouzard (line 49) is better as Madu 2009 JVI or a more recent review

The data does not really directly address cleavage by furin, rather the use of a "furin cleavage site" so the authors should be careful of wording – e.g. line 73. The data on the use of 'furin inhibitor' is not convincing, and the furin inhibitor used in not especially specific for furin; it may be best to eliminate these data if more convincing inhibition cannot be obtained (by Western blot), the H5CS mutant does not seem to be inhibited at all. Care should also be taken with labeling blots to avoid mix up between SARS1 and SARS2.

This reviewer is also not especially convinced by the data on IFITM and amphotericin B, and would recommend removal of this section of the manuscript, as is not necessary for the main conclusions

The data on the ferret model are important and appear to be well done

The data reporting different S1/S2 sites in heart and spleen tissue are also important and, novel; these data could be discussed and interpreted more extensively, in the context of structural elements in the S1/S2 loop

Reviewer #2 (Remarks to the Author):

Peacock and colleagues investigated the role of the furin cleavage motif at the S1/S2 site of the spike protein of SARS-CoV-2 in viral cell tropism and transmission. In brief, they show that "pre-cleavage" by furin at the S1/S2 is required for TMPRSS2 dependent entry but dispensable for cathepsin L dependent entry. Moreover, they provide evidence that TMPRSS2 dependent entry protects against the antiviral activity of IFITM3. Finally, they show that the furin cleavage site is required for efficient SARS-CoV-2 transmission in the ferret model.

The concept that the furin cleavage site is required for TMPRSS2 dependent entry into lung cells but not for cathepsin L dependent entry into, for instance, Vero cells has been previously documented by several studies. This finding is confirmed here and moderately extended. In contrast, the finding that TMPRSS2 dependent entry protects SARS-CoV-2 entry from inhibition by IFITM3, although not unexpected, is new. Similarly, the finding that the furin cleavage site is required for viral transmission is novel and of significant interest. The following points should be addressed.

Major

As stated above, the finding that pre-cleavage of SARS-CoV-2 spike by furin is required for subsequent TMPRSS2- but not cathepsin L-dependent virus-cell fusion has been previously demonstrated both with surrogate systems and with authentic virus, both with cell lines and primary cells. These studies should be cited, their main findings introduced in the introduction section and discussed in the discussion section. See: PMID: 32703818, PMID: 32376634, PMID: 32362314

The concept that pre-cleavage at the S1/S2 site is required for TMPRSS2-dependent entry into lung cells is based on the published finding that Calu-3 and HAE lung cells express good levels of TMPRSS2 but only low levels of cathepsin L (PMID: 27791014). The present study reports the opposite findings but provides no explanation why deletion of the furin cleavage site reduces Calu-3/HAE entry although it is dispensable for cathepsin L usage and high levels of cathepsin L are available for S protein activation in these cells.

Minor

All y-axes are labelled RLU. Some y-axes go up to 100.000 others end at 1 although presumably the same assay has been performed. Please explain.

It must be indicated in all figure legends whether the results of an independent experiment or the average of several independent experiments is shown. In the former case, please state how many confirmatory experiments were conducted. In the latter case, please state how many experiments were averaged. Please indicate whether error bars indicate SD or SEM.

"The furin cleavage site of SARS-CoV-2 mediates entry into mucosal epithelial and primary human airway cells." The spike protein mediates entry, not the furin cleavage site.

The concept that TMPRSS2 rescues CoV entry from inhibition by IFITM3 has been documented and the authors might want to cite this study (PMID: 23536651).

Reviewer #3 (Remarks to the Author):

Peacock and colleagues convincingly show that furin-mediated cleavage of the spike Protein can affect SARS-CoV-2 entry pathways in physiologically relevant cells and is exploited, in combination with TMPRSS2, to circumvent an intrinsic and interferon-inducible antiviral mechanism. Inhibition of TMPRSS2 through pharmacologic means may thus represent an effective means to reduce virus replication and promote virus clearance through the natural antiviral responses of the host.

The main conclusions of the paper are well supported by the presented data. Although I am not an expert in coronavirus biology, the experiments were clearly designed carefully and well executed from a virologist point of view.

The only experiment I would have considered worthwhile performing (but not essential for the publication of the paper) is to engineer Vero E6 cells (similar to the experiments done for 293T cells) so that they no longer select against the polybasic insertion within the SARS-CoV-2 spike protein. This would close the loop of the proposed model.

Maybe the authors could comment in their discussion on the implications of their findings on possible consequences (the threat) arising from the recent outbreaks in ferret farms throughout Europe.

Reviewer #4 (Remarks to the Author):

This comprehensive study by Peacock et al demonstrates that the SARS-CoV-2 cleavage polybasic site can be cleaved by furin, is differentially selected for depending on the expression of TMPRSS2, and that viruses lacking the furin CS are attenuated in ferrets and non-transmissible. Importantly, analysis of 100,000 genomes highlights that cleavage site deletions can naturally arise at a very low level. The manuscript is well-written and the data is compelling providing important new information. Specific comments for consideration:
1. The author's demonstrate that the expression of SARS-CoV-2 entry factors differs amongst the cells. However, the levels of cellular proteases, surface protein expression and antiviral responses can vary by cellular differentiation state and by donor. It would be important to demonstrate that similar results are obtained using differentiated Caco-2 and Calu-3 cells, which more closely model in vivo, and using at least n = 3 HAE donors.
2. Several protease inhibitors were used to show that entry is dependent on specific cellular protease. These can be associated with cytotoxicity. The author's should comment if there was increased cell death in the SARS cells.
3. Lines 175 - 176 concludes that camostat abrogates deltaCS and WT virus replication in HAEs. Yet, Figure 3D suggests that WT virus replication is delayed. Please discuss.
4. Lines 215 - 218 conclude that IFITM3 impacts viral entry yet there is no information on how this experiment was performed, the controls included, and the methods lack experimental details.

Minor
1. Line 147 should be Figure 2H.
2. Figure 2B suggests that the CS mutant virus only outcompetes WT virus in Vero cells. Do the author's think this is due to the lack of interferon?
3. Figure 4B suggests that AmphoB increases SARS replication in many cell lines, but VSV-G is inhibited in 293T-ACE2. I'm curious why this is happening.

Author Rebuttal to Initial comments

Reviewers Comments:

Reviewer #1 (Remarks to the Author):

This is a timely and important study, in what is currently an active area of SARS2 research – the function of the S1/S2 cleavage site. The work is well done

Some of the referencing in the Introduction is incomplete or inaccurate – Holshue (line 36) does not directly address the statement concerning WHI, and Belouzard (line 49) is better as Madu 2009 JVI or a more recent review

We thank the reviewer for their comments on the manuscript. As suggested by the reviewer, the highlighted references have been replaced or supplemented with more appropriate ones.

The data does not really directly address cleavage by furin, rather the use of a “furin cleavage site” so the authors should be careful of wording – e.g. line 73. The data on the use of ‘furin inhibitor’ is not convincing, and the furin inhibitor used in not especially specific for furin; it may be best to eliminate these data if more convincing inhibition cannot be obtained (by Western blot), the H5CS mutant does not seem to be inhibited at all. Care should also be taken with labeling blots to avoid mix up between SARS1 and SARS2.

We agree with the reviewer and have subsequently removed figures and assays involving furin inhibitors. We believe the evidence that furin is responsible for this cleavage is now more convincingly and extensively described by others in the literature and have cited those papers. We have also relabelled our blots to clearly indicate which samples are SARS-CoV and which SARS-CoV-2 (see Figure 1a).

This reviewer is also not especially convinced by the data on IFITM and amphotericin B, and would recommend removal of this section of the manuscript, as is not necessary for the main conclusions

We disagree with the reviewer on this point as we feel that this data is important for describing the mechanism of why the S1/S2 cleavage site is important to SARS-CoV-2 replication and we note that reviewers #2 and #3 also appear to find this result important and key to the manuscript. However, we have now bolstered these sections with new data and references to additional studies which confirm our conclusions. Notably, we have now performed live virus infections in Calu-3 cells in presence and absence of amphotericin and observed a 4-log increase in viral titre of ΔCS virus with Amphotericin B (Figure 3h). This observation strongly supports that the lack of ΔCS virus replication in Calu-3 is accounted for by a strong inhibition of its endosomal entry pathway by the IFITM2 or 3 proteins that are targeted by the drug. We also repeated HAE experiments with Amphotericin B from 2 additional donors and found a consistent phenotype. Furthermore, we have added a short discussion of a recent paper which supported our conclusion and has shown that the protein responsible for this phenotype is IFITM2 (line 252-254).

The data on the ferret model are important and appear to be well done

The data reporting different S1/S2 sites in heart and spleen tissue are also important and, novel; these data could be discussed and interpreted more extensively, in the context of structural elements in the S1/S2 loop

Although, we agree that the exact mutations seen may be of interest, we are cautious that with such a small number of different autopsy samples used, we would not wish to over-interpret our data. Any deletion of the S1/S2 cleavage site likely abrogates furin cleavage and thus our only conclusion is that these mutations do arise at low levels in tissues of infected individuals. We have however, added a short

sentence discussing how we believe that all the deletions described in this study likely have identical phenotypes in our pseudovirus assays (see lines 238-241).

Reviewer #2 (Remarks to the Author):

Peacock and colleagues investigated the role of the furin cleavage motif at the S1/S2 site of the spike protein of SARS-CoV-2 in viral cell tropism and transmission. In brief, they show that "pre-cleavage" by furin at the S1/S2 is required for TMPRSS2 dependent entry but dispensable for cathepsin L dependent entry. Moreover, they provide evidence that TMPRSS2 dependent entry protects against the antiviral activity of IFITM3. Finally, they show that the furin cleavage site is required for efficient SARS-CoV-2 transmission in the ferret model.

The concept that the furin cleavage site is required for TMPRSS2 dependent entry into lung cells but not for cathepsin L dependent entry into, for instance, Vero cells has been previously documented by several studies. This finding is confirmed here and moderately extended. In contrast, the finding that TMPRSS2 dependent entry protects SARS-CoV-2 entry from inhibition by IFITM3, although not unexpected, is new. Similarly, the finding that the furin cleavage site is required for viral transmission is novel and of significant interest. The following points should be addressed.

Major

As stated above, the finding that pre-cleavage of SARS-CoV-2 spike by furin is required for subsequent TMPRSS2- but not cathepsin L-dependent virus-cell fusion has been previously demonstrated both with surrogate systems and with authentic virus, both with cell lines and primary cells. These studies should be cited, their main findings introduced in the introduction section and discussed in the discussion section. See: PMID: 32703818, PMID: 32376634, PMID: 32362314

As suggested by this reviewer additional reference have been added and statements altered to reflect that others have also made a link between furin cleavage site and TMPRSS2 entry. (lines 51, 55, 81, 97, 249)

The concept that pre-cleavage at the S1/S2 site is required for TMPRSS2-dependent entry into lung cells is based on the published finding that Calu-3 and HAE lung cells express good levels of TMPRSS2 but only low levels of cathepsin L (PMID: 27791014). The present study reports the opposite findings but provides no explanation why deletion of the furin cleavage site reduces Calu-3/HAE entry although it is dispensable for cathepsin L usage and high levels of cathepsin L are available for S protein activation in these cells.

It is interesting that the exact levels of TMPRSS2 and cathepsin L mRNA expression we have measured in Calu 3 and HAE cells differ slightly from those reported by Park et al in the PNAS/MERS activation paper, although the patterns are the same so we do not think these are opposite findings. However, both ourselves and Park et al find that airway derived cells express cathepsin L mRNA so the endosomal route of entry is not precluded. However, we hypothesize that if TMPRSS2 is expressed and available this is a more advantageous route of entry for the virus, and will occur first anyway before the endosomal cathepsin dependent route. Our proposed mechanism is that surface entry via TMPRSS2 allows avoidance of endosomal IFITM proteins such as IFITM2/3, which we show are abundant in these cell types and that endosomal entry into these cells is largely restricted due to these proteins. We have expanded our discussion to clarify that this is our mechanism – see lines 249-254.

Minor

All y-axes are labelled RLU. Some y-axes go up to 100.000 others end at 1 although presumably the same assay has been performed. Please explain.

All assays have had RLU normalised to 1 for the control (either empty vector controls or no drug controls) – therefore we have shown axis going up to 1 for inhibitory assays or going upwards (ie up to 100) for assays where RLU has increased relative to controls. We have now clarified this in the figure legends.

It must be indicated in all figure legends whether the results of an independent experiment or the average of several independent experiments is shown. In the former case, please state how many confirmatory experiments were conducted. In the latter case, please state how many experiments were averaged. Please indicate whether error bars indicate SD or SEM.

More information on repeats and representative data is now included in figure legends throughout the manuscript. In addition, we have updated the figure legends to clarify what is plotted on each graph (mean + SD throughout).

“The furin cleavage site of SARS-CoV-2 mediates entry into mucosal epithelial and primary human airway cells.” The spike protein mediates entry, not the furin cleavage site.

This figure title has been amended to “Figure 2. The furin cleavage site of SARS-CoV-2 spike enhances entry into mucosal epithelial and primary human airway cells” to correct this.

The concept that TMPRSS2 rescues CoV entry from inhibition by IFITM3 has been documented and the authors might want to cite this study (PMID: 23536651).

As suggested we have now acknowledged this reference with a short sentence in lines 171-173

Reviewer #3 (Remarks to the Author):

Peacock and colleagues convincingly show that furin-mediated cleavage of the spike Protein can affect SARS-CoV-2 entry pathways in physiologically relevant cells and is exploited, in combination with TMPRSS2, to circumvent an intrinsic and interferon-inducible antiviral mechanism. Inhibition of TMPRSS2 through pharmacologic means may thus represent an effective means to reduce virus replication and promote virus clearance through the natural antiviral responses of the host.

The main conclusions of the paper are well supported by the presented data. Although I am not an expert in coronavirus biology, the experiments were clearly designed carefully and well executed from a virologist point of view.

The only experiment I would have considered worthwhile performing (but not essential for the publication of the paper) is to engineer Vero E6 cells (similar to the experiments done for 293T cells) so that they no longer select against the polybasic insertion within the SARS-CoV-2 spike protein. This would close the loop of the proposed model.

As suggested by this review we performed head to head live virus growth curves in Vero and Vero cells engineered to expressed TMPRSS2 cells. In the TMPRSS2-expressing cells we find the growth advantage of the delta CS virus is abrogated and indeed the wt is now the fitter virus, as seen in other cells that expressed abundant TMPRSS2 (see Figure 3b,c and lines 161-167).

Maybe the authors could comment in their discussion on the implications of their findings on possible consequences (the threat) arising from the recent outbreaks in ferret farms throughout Europe.

As suggested, we have now added a short line and several references about the European mink outbreaks showing ferret/mink are highly susceptible to sars-cov-2 infection. (lines 193-195), although we do not expect selection around the furin cleavage site in these zoonotic situations.

Reviewer #4 (Remarks to the Author):

This comprehensive study by Peacock et al demonstrates that the SARS-CoV-2 cleavage polybasic site can be cleaved by furin, is differentially selected for depending on the expression of TMPRSS2, and that viruses lacking the furin CS are attenuated in ferrets and non-transmissible. Importantly, analysis of 100,000 genomes highlights that cleavage site deletions can naturally arise at a very low level. The

manuscript is well-written and the data is compelling providing important new information. Specific comments for consideration:

1. The author's demonstrate that the expression of SARS-CoV-2 entry factors differs amongst the cells. However, the levels of cellular proteases, surface protein expression and antiviral responses can vary by cellular differentiation state and by donor. It would be important to demonstrate that similar results are obtained using differentiated Caco-2 and Calu-3 cells, which more closely model in vivo, and using at least n = 3 HAE donors.

As suggested by the reviewer, to investigate donor variation, we have repeated several key experiments in additional HAE donors (N=3, see figure 2h and 3i). Furthermore, we have performed an additional experiment in Calu-3 with amphotericin B, which showed an identical phenotype to the same experiment performed in HAEs from different donors (Figure 3h). In addition we have now measured mRNA expression of the relevant host factors in HAE from 3 different donors (figure 2h) as well as in Calu 3 and Caco2 (figure 2f and g). We appreciate the reviewer's suggestion of using differentiated Caco-2 and Calu-3 cell lines but we are already using HAE cells which are highly differentiated and are the closest cell model to in vivo viral targets. We have also shown that non-differentiated versions of Caco-2 and Calu-3 show identical phenotypes to fully differentiated human airway epithelial cells from multiple different donors.

2. Several protease inhibitors were used to show that entry is dependent on specific cellular protease. These can be associated with cytotoxicity. The author's should comment if there was increased cell death in the SARS cells.

We agree a possible explanation for the high potency of camostat could have been cell death. Therefore, as suggested by the reviewer, we tested the viability of primary airway cells in the presence of the protease inhibitor camostat as used in figure 2d by measuring transepithelial electrical resistance (TEER). We show no loss of membrane integrity suggesting camostat is not causing cytotoxicity, this data is shown in supplementary figure 1a and referenced in the main text (Line 143-145).

3. Lines 175 - 176 concludes that camostat abrogates deltaCS and WT virus replication in HAEs. Yet, Figure 3D suggests that WT virus replication is delayed. Please discuss.

We have altered the wording of this line (now line 142) to reflect that camostat delays, rather than fully inhibits replication.

4. Lines 215 - 218 conclude that IFITM3 impacts viral entry yet there is no information on how this experiment was performed, the controls included, and the methods lack experimental details.

We apologise for this oversight – a detailed section has now been included in the methods of how these experiments were performed (lines 378-382).

Minor
1. Line 147 should be Figure 2H.

This figure has been removed in our revised manuscript along with the relevant text.

2. Figure 2B suggests that the CS mutant virus only outcompetes WT virus in Vero cells. Do the author's think this is due to the lack of interferon?

This is now figure 1e. Although lack of interferon may contribute (as we don't think IFITM proteins would be very highly expressed in these cells), we believe that this phenotype is mostly due to lack of TMPRSS2 expression as we see a similar phenotype with pseudovirus in 293T-ACE2, which potentially can produce and react to interferon. Furthermore, the new figures 3b and c, suggest that when TMPRSS2 is expressed in Vero cells this phenotype reverses further indicating TMPRSS2 is the main determinant of this phenotype in every cell type tested.

3. Figure 4B suggests that AmphoB increases SARS replication in many cell lines, but VSV-G is inhibited in 293T-ACE2. I'm curious why this is happening.

We are unsure why VSV-G is inhibited – we consistently see a larger inhibition of VSV-G than MLV-A with amphotericin B, which implies it's not a cytotoxicity effect but rather a specific effect to VSV-G. This effect doesn't change our conclusions as we don't see an effect with MLV-A. We included a pair of controls in all these

Decision Letter, first revision:

Dear Wendy,

Thank you for submitting your revised manuscript "The furin cleavage site of SARS-CoV-2 spike protein is a key determinant for transmission" (NMICROBIOL-20103190A). It has now been seen by 3 out of 4 original referees and their comments are below. Due to the fast pace of this field we proceeded with a decision, although one report was still missing. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Microbiology, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Microbiology Please do not hesitate to contact me if you have any questions.

Congratulations and you will hear from me soon with the checklist!

Reviewer #2 (Remarks to the Author):

The authors have adequately addressed all points raised by this reviewer. The manuscript is of substantial interest to the field.

Two minor points:

"...improved by amphoB pre-treatment, showing that all PVs entered these cells through endosomes(Figure 3b). Conversely, in Caco-2 and Calu-3 cells, entry of PVs with uncleaved spikes was boosted by amphoB treatment, whereas there was little or no effect on the entry of PVs with furin CScontaining spikes (Figure 4c,d)." The references to the figures seem to be incorrect.

In figures 4c-d, the "a", "b", "c" and "d" appearing out of context must be deleted.

Reviewer #3 (Remarks to the Author):

All my comments have been comprehensively addressed.

Reviewer #4 (Remarks to the Author):

The author's were responsive to reviewer's comments. No further concerns.

Decision Letter, final checks:

Dear Wendy,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Microbiology manuscript, "The furin cleavage site of SARS-CoV-2 spike protein is a key determinant for transmission" (NMICROBIOL-20103190A). Please carefully follow the step-by-step instructions provided in the personalised checklist attached, to ensure that your revised manuscript can be swiftly handed over to our production team.

Due to the timeliness of your work, we would like to receive your revised paper, with all of the requested files and forms, ideally within 5 business days, by 18th March 2021. I appreciate it is a lot of work within a short time-frame, but this will enable us to pass it to the production team without delays. Please get in contact with us if you anticipate delays, and provide us with an estimate regarding when you will submit these files.

When you upload your final materials, please include a point-by-point response to any remaining reviewer comments.

If you have not done so already, please alert us to any related manuscripts from your group that are under consideration or in press at other journals, or are being written up for submission to other journals (see: https://www.nature.com/nature-research/editorial-policies/plagiarism#policy-on-duplicate-publication for details).

In recognition of the time and expertise our reviewers provide to Nature Microbiology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "The furin cleavage site of SARS-CoV-2 spike protein is a key determinant for transmission". For those reviewers who give their assent, we will be publishing their names alongside the published article.

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If you have any further questions, please feel free to contact me.

Reviewer #1:
None

Reviewer #2:
Remarks to the Author:
The authors have adequately addressed all points raised by this reviewer. The manuscript is of substantial interest to the field.

Two minor points:

"...improved by amphoB pre-treatment, showing that all PVs entered these cells through endosomes(Figure 3b). Conversely, in Caco-2 and Calu-3 cells, entry of PVs with uncleaved spikes was boosted by amphoB treatment, whereas there was little or no effect on the entry of PVs with furin CScontaining spikes (Figure 4c,d)." The references to the figures seem to be incorrect.

In figures 4c-d, the "a", "b", "c" and "d" appearing out of context must be deleted.

Reviewer #3:
Remarks to the Author:
All my comments have been comprehensively addressed.

Reviewer #4:
Remarks to the Author:
The author's were responsive to reviewer's comments. No further concerns.

Final Decision Letter:

Date: 8th April 21 11:48:42
Last Sent: 8th April 21 11:48:42
Triggered By: Julie Tai-Schmiedel
From: julie.tai-schmiedel@nature.com
To: w.barclay@imperial.ac.uk
CC: communities@nature.com
BCC: rjsproduction@springernature.com;rjsart@springernature.com
Subject: Decision on Nature Microbiology manuscript NMICROBIOL-20103190B
Message: 8th April 2021

Dear Wendy,

Thank you and your co-authors for providing the requested adjustments of your manuscript. I am now pleased to accept your Article "The furin cleavage site in the SARS-CoV-2 spike protein is required for transmission in ferrets" for publication in Nature Microbiology. Thank you for having chosen to submit your work to us and many congratulations.

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Congratulations again and I look forward seeing your paper published!

With kind regards,

Julie

Julie Tai-Schmiedel
Editor
Nature Microbiology