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TMPRSS2 expression dictates the entry route used by SARS-CoV-2 to infect host cells

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1st Editorial Decision 25th Feb 2021

Dear Dr. Lozach,

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see from the comments below, the manuscript received a bit of a mixed response. Some issues are raised regarding overall novelty and referee #3 is not convinced that the analysis has been taken far enough to consider publication here. However, as you can also see referees #1 and 2 are more supportive of the study. I am in agreement with this and find that the study makes an important contribution and I would like to invite a revision.

It would be good to discuss how to best address the referees' comments and we can do so via video or email whatever works best for you.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
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Thank you for the opportunity to consider your work for publication. I look forward to discussing your revisions further

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
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Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

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- a word file of the manuscript text.

- individual production quality figure files (one file per figure)
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Referee #1:

Koch et al. investigated the role of proteases TMPRSS2 and cathepsin L and vesicular acidification in SARS-CoV-2 entry routes. They selected four permissive cell lines, Caco2 and Calu-3 which are TMPRSS2 positive and A549-ACE2 and Vero which are TMPRSS2 negative. The compared in these cells the effect of various protease inhibitors and drugs that modulate vesicular acidification. Studies are performed at 8h post infection which they show is equivalent to one round of infection. Overall, they show that entry into TMPRSS2+ cells is faster and is inhibited by TMPRSS2 inhibiting drugs while entry in TMPRSS2- cells is slower and inhibited by cathepsin L and endosomal acidification inhibiting drugs. They also studied the role of acidification on SARS-CoV-2 spike mediated viral entry and cell-cell fusion. Experiments are well performed and demonstrate how SARS-CoV-2 may fuse at the plasma membrane or after internalization in different cell types

Major comments
1. The authors compared four unrelated cell lines and made a correlation between viral entry and the presence of TMPRSS2. The differences may be mediated by other variations between the cells. It would be more convincing to add results in the same line expressing or not TMPRSS2 (either overexpress TMPRSS2 in a negative cell or silence or knock down TMPRSS in a positive cells
2. Drug toxicity in the different cell lines is not addressed.
3. data are normalised to the control and there are no representative dot plots for the FACS. It is stated in the text that the aim was to obtain about 20% infection, which suggests different MOIs for each cell line? It would be nice to specify in the text the MOIs used for each cell line and see some of the non-normalised graphs and FACS plots, at least for figure s2 A & B.
4. Fig. 1 TMPRSS2 western blot image quality is low.
5. Fig 2, (see point 1.) t would have been nice to have a complementary approach such as

overexpression of TMPRSS2 or knock down of cathepsin L / TMPRSS2 in this system to confirm the inhibitor-based approach.Does knock-down of cathepsin L in vero or A549* cells or knock-down of TMPRSS2 in Calu-3 or Caco-2 inhibit viral entry as shown with aprotonin? Does overexpression of TMPRSS2 in vero or A549* cells revert inhibition of entry by cathepsin L inhibitor SB412515?
6. Fig. 3. The dose of NH4Cl in figure 3 E is 3 times higher than the maximal dose in figure A, why was such a high dose chosen? Is this why it might have a non-specific effect on cells leading to absence of infection?
7. Fig. 3 Bafilomycin A1 also inhibits infection in Calu3 and Caco2 cells, which are TMPRSS2+. This does not fit with the model of "mutually exclusive entry routes" presnetde in the abstract and text
8. The dominant negative and constitutively active Rab7a only have a minor inhibitory effect on the graph compared to control (20-30%) while overexpression of WT Rab7a increases fusion.
9. Cell-cell fusion experiments. The authors should stain the cells (S or N) to show that the syncytia are productively infected Bi-nucleated cells may correspond to cells undergoing division, are many 3+ nuclei cells observed in this assay? There is no evidence of fusion here as there is no marker of cytoplasmic or membrane mixing.

Referee #2:

SARS-CoV-2 infects cells by binding to the host cell receptor ACE2, through its Spike glycoprotein present on the envelope of the viral particle, which triggers virus-host membrane fusion. Previous studies have show that CoV2 can be cleaved by TMPRSS2 at the plasma membrane or cathepsin L in the endosome, in order to facilitate infection. Most of this work has been carried out using inhibitors of these proteases and in the present manuscript Koch et al. take the same approach and reach similar conclusions. Endosomal acidification is known to be important for cathepsin L mediated entry (eg https://pubmed.ncbi.nlm.nih.gov/33465165/), as first shown for SARS-CoV-1 in 2005 (https://pubmed.ncbi.nlm.nih.gov/16081529/). The data by Koch et al. agrees with this previous work.

Major Points:

1. The authors claim that the Vero cells are TMPRSS2 negative but some cell-cell fusion events can still be detected when they are infected with SARS-CoV-2. The authors should provide an explanation for this, as the small amount of TMPRSS2 present in Vero cells can influence the comparison with other cell lines in infection experiments in the manuscript.
2. The western blot in Figure 1A which compares the TMPRSS2 levels in cell lines is not convincing. The actin in Caco2 lane is roughly the double that of Vero and A549* and so is TMPRSS2; this raises doubts about the actual amount of TMPRSS2 in those cell lines. The authors should provide a new western blot loading the same amount of cell lysate and show quantification analysis of TMPRSS2 for each cell line.
3. The use of inhibitors to dissect the different ways of entry and fusion is not ideal for this type of study due to off-target activity or incomplete inhibition of the targeted protease, which can alter downstream results. In particular, the Aprotonin used in this study may block not only TMPRSS2 but also other serine proteases (e.g. TMPRSS4) which can be involved in the entry process. Genetic knockout (eg. TMPRSS2 KO) should be used to confirm the results and examine the exact contribution of TMPRSS2 protease in SARS-CoV-2 entry.
4. It is concerning that different MOIs are used for the different cell lines, and are not consistent between different experiments (Fig.1C and 1D). Furthermore, different inhibitors concentrations were used for different cell lines. It is important to be consistent throughout the assays or clarify in

the text why different MOIs and drug concentrations were used and how they were determined (see minor comments for details).
5. The experiments exploring the role of furin in processing and activating Spike protein and its role in cell-cell fusion are also concerning. In figure 6, the virus was produced in cells expressing furin. The effect of furin cannot be truly quantified as all the newly produced particles already contain a pre-cleaved Spike at the S1-S2 site. The same issue is true for the contribution of furin cleavage to the cell-cell fusion. Most of the Spike protein produced by infected cells and exposed on the cell surface to mediated cell-cell fusion is already cleaved, so adding furin to the medium may not have any detectable effect, leading to a misleading conclusion. This experiment should be run in Vero cells knocked out for furin and then adding the furin enzyme to the medium after infection.

Minor comments:

1. Line 116: "The susceptibility of Caco-2 and Vero cells to SARS-CoV-2 at multiplicities of infection (MOIs) of 0.1 and 0.5" Why different MOIs were used for the two cell lines? How was this assessed?
2. Line 119: "Similarly, 35% of Vero cells were infected at 8 hpi (Fig. 1C)." As the number of Vero cells showed in the panel is lower than in Caco2, at least two fields for each cell line with the same number of cells is needed. Including quantification analysis would be beneficial.
3. Why the MOI used in Fig.1C is 0.1 for Caco2 and 0.5 for Vero cells, while for Fig.1D the difference in MOI is 0.3 for Caco2 and 0.003 for Vero cells? And why the percentage of infected Vero cells in D is only slightly higher than in C although the MOI used in D is around 100 times less?
4. Line 205: "NH4Cl disrupts TMPRSS2+ cell-specific functions that are important for SARS-CoV-2 replication". The concentration of NH4CL used may also impact on the replication step in these cell lines. Can the authors perform a NH4CL titration to find the right concentration where entry is inhibited but replication is not affected? Also, the replication is not compromised in Fig.3A where Caco2 showed around 20 % of infected cells in the presence of the NH4Cl. How the authors explain this difference in the two assays?
5. Have the authors considered to test Chloroquine rather than NH4Cl (see https://pubmed.ncbi.nlm.nih.gov/33465165/)?
6. Line 203: "detectable with 17% of Calu-3 and Caco-2 cells infected (data not shown)". Please include these data to allow a comparison.
7. Line 281: "as described for unrelated viruses". The authors should mention that SARS-CoV-2 Spike protein was already demonstrated to trigger cell to cell fusion and provide references of the studies.
8. The fusion assay used in Fig.5 to look at cell-cell fusion in different pH conditions should be improved for clarity. Since the detection of a small number of fusion events (in Vero cells) may be rather difficult in this assay, have the authors considered establishing a dual color assay or a fluorescent protein-based assay (e.g. Split-GFP based system)? This would allow a more precise quantification of the fusion events and the impact of pH in Spike mediated cell-cell fusion process.
9. Line 433: "...trimers cut by furin." Recent study showed that furin is not the only enzyme processing the spike at the S1-S2 site. This should be stated, and references should be provided.
10. Figure 1E: Can the authors explain why they chose to use MFI rather than the same quantification assay as in D (% of NP positive cells)? It would be better to be consistent through the experiments.
11. Figure 2A: The use of Aprotonin may block other serine proteases (TMPRSS4, TMPRSS11D, TMPRSSD11E) which have been showed to play a role in SARS-CoV-2 S priming (https://doi.org/10.1038/s41598-020-78402-2). A genetic knockout of the TMPRSS2 protease in TMPRSS2+ Caco2 and Calu3 cells is needed to dissect the exact role of TMPRSS2 protease in SARS-CoV-2 entry.
12. Figure 3E: NH4Cl concentration used is 50mM for A549 and Vero and 75mM Calu-3 Caco-2.

Why there is this difference compared to the concentrations tested in Fig 3A? It would be better to be consistent through the assays.
13. Figure 3F: Why Calu3 and A549* were omitted from this graph? Please include them.
14. Figure 4F: Why different concentrations of MG132 were used between Calu3 and Caco2? This may be confusing; it would be clearer to show the same concentration range for both cell lines. How the authors explain the different sensitivity to MG132 between Caco2 and Calu3 at 3.7 uM? Please clarify.
15. Figure 4G: Please clarify why 60µM MG132 was used in this experiment instead of using the concentrations tested in 4F.
16. Figure 5C: Please provide data using viral protein staining (Nucleoprotein or Spike) to prove that the fused cells are infected. Why does cell-cell fusion occur in the absence of TMPRSS2? The fact that cell-cell fusion is directly correlated to TMPRSS2-mediated cleavage of Spike and is still detected in Vero cells in absence of trypsin suggests that certain levels of TMPRSS2 are still present in this cell line, which will also influence infection data in the study.
17. Figure 6A: Was this virus produced in presence or absence of serum (It is not specified in M&M section)? If the virus was amplified and resuspended in presence of serum, this may inactivate part of the trypsin added, underestimating the infection data.
18. Figure 6A-B: Was the virus produced in cells expressing furin? If so, the effect of furin cannot be quantified as most of the particle produced have an already pre-cleaved Spike. A western blot to show the S cleavage on virions would give the exact amount of cleaved spike present before and after furin treatment.
19. Figure 6B: Please provide a staining with a viral protein to detect the infected cells.
20. Figure 6C: See major comment on furin (last point)

Referee #3:

Koch et al. ('Host Cell Proteases Drive 1 Early or Late SARS-CoV-2 Penetration') set out to determine if SARS-CoV-2 enters epithelial cells from intracellular compartments or the plasma membrane, and how acidic pH contributes to the early steps of infection. They use different epithelial cell lines commonly used in SARS-CoV-2 research (Vero, A549, Calu-3, Caco-2), either expressing or not expressing the SARS-CoV-2 activating trypsin-like protease TMPRSS2. They confirm that TMPRSS2 is an essential host factor for SARS-CoV-2 infection. Evidence is largely based on chemical inhibitors, such as aprotinin, SB142515, MG132, and a range of acidification inhibitors.

The study is, unfortunately, somewhat shallow and incomplete. It remains descriptive, and does not elucidate what function these putative entry pathways would have for SARS-CoV-2. It is unclear if the virus penetrates from the plasma membrane or an early endosomal vesicle in TMPRSS2 positive cells. Cell biological correlate analyses of trafficking or drug controls are largely lacking. This is a pity as it might lead the authors to discover a mechanism, for example towards the question what prevents the virus from being endocytosed in cells expressing the activating protease TMPRSS2.

Major points:
1) The title is misleading as it suggests that SARS-CoV-2 uses early endosomes for infection. The manuscript does not furnish unequivoval evidence for this claim. In TMPRSS2 expressing cells, plasma membrane fusion cannot be excluded.

2) Authors use inhibitor profiling experiments across different cell lines, for example aprotinin, SB412515, NH_4Cl, BafA1, colcemid. I find it a bit daring to compare different cell lines across inhibitor applications, and then try to conclude on a mechanism of action considering whether or not the different cell lines express the TMPRSS2 protease. To me a safer approach would be to prepare TMPRSS2 KO or gain of function cells, and compare their drug susceptibilities to the parental cells.

3) It is curious that NH_4Cl inhibited SARS-CoV-2 infection of Calu-3 and Caco-2 cells irrespective of the time of addition. Authors speculate that this is due to acidification of a compartment used in virus egress. Can they be more specific here, and support this speculation with experimentation? Did they do wash out experiments of NH_4Cl?

4) They use Rab7a overexpression in transfected Vero cells to test in a late step in endosome maturation was involved in SARS-CoV-2 infection. Mutant Rab7a (active or inactive) reduced infection by about 20%. Such transfections have systemic effects on cells, besides the endo-lysosomal pathway. A minor reduction of infection as reported here could easily be due to noise in the assay. These data do not convincingly support the importance of an early to endosome trafficking step in SARS-CoV-2 infection.

5) Colcemid treatment reduced Vero infection by some 40%. This is a rather weak effect to build a scenario implying an early to late endosome trafficking step in infection. And the authors suggest that TMPRSS2 pos cells (Caco2) also use some late endosome processsing steps for infection since these cells are also somewhat sensitive to colcemid, albeit the effect is even weaker than in Vero cells. This claim stands on very thin ice.

6) The proteasomal inhibitor MG132 reduced Vero infection. The authors speculate that this is due to depletion of free ubiquitin. Again, quite a daring conclusion in the absence of additional data. More experiments are required to support this claim.

Other points:
7) Line 61: I think this statement is not entirely correct as viruses also transmit to other cells by formation of syncytia without using their envelope.

8) Line 70: would is 'act only once'?

9) How did they define moi?

10) Fig2 A,B,C; Fig 4A, D; Fig6A; Fig7C: How can an infection exceed 100%? I am lost.

11) Does the rt-qPCR assay measure subgenomic RNA as well? This matters as different cell lines may produce different subgenomic RNAs.

12) Is aprotinin a peptidic protease inhibitor taken up into cells, and acting in endosomal vesicles?

Peer Referee #1 comments

Koch et al. investigated the role of proteases TMPRSS2 and cathepsin L and vesicular acidification in SARS-CoV-2 entry routes. They selected four permissive cell lines, Caco2 and Calu-3 which are TMPRSS2 positive and A549-ACE2 and Vero which are TMPRSS2 negative. The compared in these cells the effect of various protease inhibitors and drugs that modulate vesicular acidification. Studies are performed at 8h post infection which they show is equivalent to one round of infection. Overall, they show that entry into TMPRSS2+ cells is faster and is inhibited by TMPRSS2 inhibiting drugs while entry in TMPRSS2- cells is slower and inhibited by cathepsin L and endosomal acidification inhibiting drugs. They also studied the role of acidification on SARS-CoV-2 spike mediated viral entry and cell-cell fusion. Experiments are well performed and demonstrate how SARS-CoV-2 may fuse at the plasma membrane or after internalization in different cell types

Author response:

We first want to thank Reviewer 1 for having taken the time to evaluate our manuscript and for their positive comments. The line numbers in our responses refer to the **Word file** of the revised manuscript, without any track changes.

Major comment 1. The authors compared four unrelated cell lines and made a correlation between viral entry and the presence of TMPRSS2. The differences may be mediated by other variations between the cells. It would be more convincing to add results in the same line expressing or not TMPRSS2 (either overexpress TMPRSS2 in a negative cell or silence or knock down TMPRSS in a positive cells.

Author response:

Thanks for this suggestion. We agree with the reviewer that there could be cell specific factors changing the entry route and it is indeed important to clarify this in the same cell line. To address this comment, we obtained A549* cells that overexpress TMPRSS2, which is not expressed to a detectable level in the parental A549* cells (Fig. 4A). Using these cells, we could show that the TMPRSS2 inhibitor (camostat mesylate) blocked SARS-CoV-2 infection in A549* cells expressing TMPRSS2 in a dose-dependent manner, but not at all in parental A549* cells which

lack TMPRSS2 (Fig. 4B). Conversely, the cathepsin L inhibitor (SB412515) impaired infection only in parental cells, and not in A549* cells expressing TMPRSS2 (Fig. 4C). Additionally, TMPRSS2 expressing A549* cells SARS-CoV-2 infected became resistant to agents that elevate endosomal pH (Fig. 4D). Together, these new results support our model that SARS-CoV-2 can use distinct, mutually exclusive entry routes and that TMPRSS2 is important for sorting the virus into either pathway. The results are discussed in lines 271-289 as follow:

"*To correlate the presence of TMPRSS2 with the viral entry pathway, we examined SARS-CoV-2 infection in the same cell line expressing or lacking the protease. To this end, we stably expressed TMPRSS2 in the TMPRSS2- A549* cells and confirmed the overexpression by SDS-PAGE and western blotting (Fig. 4A) (Steuten et al., 2021). As expected, the TMPRSS2 inhibitor, camostat mesylate, did not inhibit SARS-CoV-2 infection when it was added to the parental A549* cells prior to infection (Fig. 4B). However, we found that camostat mesylate reduced SARS-CoV-2 infection in a dose-dependent manner in TMPRSS2-overexpressing A549* cells (Fig. 4B). Conversely, the cathepsin L inhibitor (SB412515), efficiently prevented infection of regular A549* cells but had no effect on infection of A549* cells that expressed TMPRSS2 (Fig. 4C).*

Our results indicated that endosomal acidification is required for SARS-CoV-2 infection of TMPRSS2- cells but not for infection of cells expressing TMPRSS2 (Fig. 3E to 3H). To evaluate the role of TMPRSS2 in the dependence of SARS-CoV-2 on low pH for entry, A549 cells expressing or lacking TMPRSS2 were infected in the presence of bafilomycin A1 to neutralize endosomal pH. As anticipated, infection of TMPRSS- A549* cells with SARS-CoV-2 decreased dramatically with increasing concentrations of bafilomycin A1 (Fig. 4D). In contrast there was no noticeable effect of bafilomycin A1 on infection of TMPRSS2+ A549* cells. This confirmed that SARS-CoV-2 infection does not rely on endosomal acidification when TMPRSS2 is expressed. Overall, TMPRSS2 appears as the major determinant of the fast pH-independent route taken by SARS-CoV-2 to enter and infect TMPRSS2+ cells.*"

Major comment 2. Drug toxicity in the different cell lines is not addressed.

Author response:

All drugs in our study were used in ranges of concentration known to have no adverse effect on cells. However, we assessed the cytotoxicity of all our drugs in the range of concentrations used

on our four cell lines using the CytoTox 96 Non-Radioactive Cytotoxicity assay (Promega), which quantitatively measures the release of lactate dehydrogenase in the supernatant upon cell death and lysis. PPMP, a ceramide analog that impairs ceramide maturation and cell membrane integrity, served as a positive control. The results are shown in Figure S5 and described in lines 167-170 as follow:

"*Of note, the protease inhibitors, and all other drugs used in this work, were evaluated in a range of concentrations for which no cytotoxicity was detected, as shown by a quantitative assay measuring the release of lactate dehydrogenase into the extracellular medium upon cell death and lysis (Fig. S5).*"

Major comment 3. data are normalised to the control and there are no representative dot plots for the FACS. It is stated in the text that the aim was to obtain about 20% infection, which suggests different MOIs for each cell line? It would be nice to specify in the text the MOIs used for each cell line and see some of the non-normalised graphs and FACS plots, at least for figure s2 A & B.

Author response:

Referee 1 is correct. We used different MOIs for each cell line with the aim of achieving about 20% infection, which generally allows us to assess the potential effects of a perturbant on infection both up and down. SARS-CoV-2 was produced from Vero cells, and supernatants were previously titered on Vero cells by TCID50 assay. Reviewer 2 also pointed out that it was insufficiently explained in the original manuscript, and furthermore, that TCID50 values cannot be used strictly speaking to define the MOI. To clarify this important point, the MOI for SARS-CoV-2 is now given according to the titer determined on Vero cells by plaque-forming unit assay, and the definition of MOI is described in the MM section, lines 644-650, as follow:

"*For titration of SARS-CoV-2, confluent monolayers of Vero cells were infected with 10-fold dilutions of virus in serum-free medium and then grown in the presence of complete medium containing 2% serum and 0.05% agarose to prevent virus spread. Plaques were stained by crystal violet three days post-infection. The MOI was assigned for SARS-CoV-2 according to the titer determined on Vero cells. The MOI for SFV and UUKV was given based on the titers determined on BHK-21 cells as previously described (Lozach et al., 2010).*"

Furthermore, we now indicate the MOIs used for each cell line in the legends of figures. In addition, we also show flow cytometry dot plots corresponding to the Figures 2A (aprotinin at 5 µM) and 2B (SB412515 at 2.5 µM) in Figures S2 and S4, respectively.

Major comment 4. Fig. 1 TMPRSS2 western blot image quality is low.

Author response:

We apologize for the poor quality of the original image. We now show new TMPRSS2 western blot images with much better quality (Fig. 1A and Fig. 4A). Please, see also our response to Major point 2 of Reviewer 2.

Major comment 5. Fig 2, (see point 1.) t would have been nice to have a complementary approach such as overexpression of TMPRSS2 or knock down of cathepsin L / TMPRSS2 in this system to confirm the inhibitor-based approach.Does knock-down of cathepsin L in vero or A549* cells or knock-down of TMPRSS2 in Calu-3 or Caco-2 inhibit viral entry as shown with aprotonin? Does overexpression of TMPRSS2 in vero or A549* cells revert inhibition of entry by cathepsin L inhibitor SB412515?

Author response:

Please, see our response to Major comment 1. Briefly, we have now added a new figure (Fig. 4) showing that TMPRSS2 overexpression in A549* cells renders SARS-CoV-2 infection no longer sensitive to cathepsin L inhibitor SB412515.

Major comment 6. Fig. 3. The dose of NH4Cl in figure 3 E is 3 times higher than the maximal dose in figure A, why was such a high dose chosen? Is this why it might have a non-specific effect on cells leading to absence of infection?

Author response:

Infection in the presence of 33 mM NH_4Cl was still readily detectable in three of our four cell lines (Fig. 3A). To accurately determine the acid-dependent half time of viral penetration (Fig. 3E), it

is essential to ensure that further penetration is prevented once the weak base is added. Therefore, we used a maximal NH_4Cl concentration of 75 mM to completely abolish infection in the add-in assay. The inhibition is now shown in Figure S6A. Of note, it is commonly accepted that up to 50-100 mM NH_4Cl is not detrimental for cells, which was confirmed, at least, in our four cell lines with our cytotoxicity assay (Fig. S5). This point is clarified in text, lines 220-222, as follow:

"*To ensure that viral penetration is completely abolished after adding NH4Cl, a concentration of up to 75 mM was used (Fig. S6A).*"

To further document the entry-independent effect of NH_4Cl on SARS-CoV-2 infection in Calu-3 and Caco-2 cells (TMPRSS2+), we performed the reverse approach of adding NH_4Cl. This assay consists of washing NH_4Cl out at different times following synchronization and the onset of infection. Results are shown in Figure S6B. They clearly indicate that NH_4Cl had no noticeable effect on SARS-CoV-2 infection in these cells for the first hour after warming, at a time when the virus had already penetrated the cells, but viral replication had not yet begun (Fig. 1F and 2C). In contrast, SARS-CoV-2 infection in A549* and Vero cells (TMPRSS2-) decreased during the first 30 min until it was no longer detectable after 2 h (Fig. 3F), indicating overall that SARS-CoV-2 cannot remain infectious for very long before acid-activated penetration into TMPRSS2- cells. The set of new results is discussed in lines 226-269 as follow:

"*To examine how long SARS-CoV-2 remains acid-activable in TMPRSS2- cells, we followed the reverse approach of adding NH4Cl. This assay relies on the fact that the neutralization of endosomal pH by NH4Cl is reversible after washing. Virus binding to Vero cells was synchronized at low MOI (~0.3) on ice, and cells were rapidly shifted to 37°C in the presence of NH4Cl before the weak base was washed out at varying times. In other words, we determined the time at which SARS-CoV-2 acid activation was no longer possible. In Vero cells, infection decreased by 70% during the first 30 min and then more slowly until it reached a 90% decrease after 2 h (Fig. S6B), which was the exact opposite of the NH4Cl addition approach (Fig. 3E). Together, the NH4Cl add-in and wash-out kinetics indicated that SARS-CoV-2 infectivity decreases sharply in TMPRSS2- cells if the virus is not allowed to enter the cytosol rapidly by acid-activated penetration, most likely, from endosomal vesicles.*

In Calu-3 and Caco-2 cells, both of which express TMPRSS2, it was not possible to determine the timing of the acid-requiring step. We failed to detect SARS-CoV-2-infected cells when NH4Cl was added, even if the addition occurred several hours after transferring the cells

from 4 to 37°C (Fig. 3E). In samples where NH4Cl was omitted, however, infection was readily detectable with 17% of Calu-3 and Caco-2 cells infected (Fig. S6A) suggesting that the weak base interferes with SARS-CoV-2 replication in these two cell lines. To clarify whether NH4Cl blocks virus entry or replication in these cells, we next assessed SARS-CoV-2 infection in our wash-out assay. Neutralization of endosomal pH with NH4Cl in Caco-2 cells had no noticeable effect on SARS-CoV-2 infection for the first hour after warming (Fig. S6B), when the virus had normally already penetrated TMPRSS2+ cells (Fig. 2D). In this assay, viral replication was not affected, most likely because the weak base was washed out early enough to avoid side effects. Altogether, these results suggest that SARS-CoV-2 does not depend on endosomal acidification for infectious entry into TMPRSS2+ cells, but rather that NH4Cl disrupts Calu-3 and Caco-2 cell-specific functions that are important for SARS-CoV-2 replication. NH4Cl not only neutralizes the intracellular pH but also alters all endosomal, lysosomal, and trans-Golgi network functions that are acid dependent (Helenius, 2013).

As an alternative method to alter endosomal pH, we added the vATPase inhibitor concanamycin B to cell-bound virus, instead of NH4Cl, at different times after warming. The time course showed that infectious entry of UUKV began later than 15 min and had not reached a maximum 2 h after cell warming. In marked contrast, SARS-CoV-2 infection was virtually insensitive to the concanamycin B addition as early as a few seconds after shifting TMPRSS2+ cells to 37°C (Fig. 3F). As expected, SARS-CoV-2 passed the concanamycin B-sensitive step in TMPRSS2- cells within less than 15 min, and infectious entry reached a plateau value after 45 min, somewhat faster than in cells treated with NH4Cl (Fig. 3G). This difference in sensitivity to endosomal pH may be because concanamycin B interferes not only with endosomal functions that are acid dependent but also, indirectly, with the maturation of endosomes. However, unlike NH4Cl, it was apparent that concanamycin B had no adverse effect on SARS-CoV-2 replication in any of these experiments. Neutralization of endosomal pH by chloroquine lead to similar kinetics of SARS-CoV-2 infection as concanamycin B (Fig. 3H).

Taken together, these results strongly suggest that SARS-CoV-2 can use two different routes to enter and infect target cells, i.e., a fast pH-independent route in TMPRSS2+ cells (Fig. 2D, 3F, S6B, and 3H) and a slow acid-activated route in cells lacking TMPRSS2 (Fig. 2C, 3E, 3G, and 3H)."

Major comment 7. Fig. 3 Bafilomycin A1 also inhibits infection in Calu3 and Caco2 cells, which are TMPRSS2+. This does not fit with the model of "mutually exclusive entry routes" presnetde in the abstract and text

Author response:

SARS-CoV-2 infection is effectively sensitive to bafilomycin A1 in Calu-3 and Caco-2 cells (TMPRSS2+), but much less than in A549* and Vero cells (TMPRSS2-). Our results with other agents that elevate endosomal pH, namely, NH_4Cl, chloroquine, and concanamycin B follow the same trend (Fig. 3A to 3D), and do not argue for a direct requirement of endosomal acidification for SARS-CoV-2 fusion and infection in TMPRSS2+ cells (Fig. 3F to 3H, and 4D).

We believe that agents that elevate endosomal pH prevent the acid-dependent SARS-CoV-2 penetration into TMPRSS2- cells but that the same agents most likely interfere with SARS-CoV-2 entry and infection not through the neutralization of endosomal pH but indirectly through alteration of all endosomal, lysosomal, and trans-Golgi network functions that are acid dependent. Our model is indeed that SARS-CoV-2 can use two different routes to enter and infect target cells, which are mutually exclusive, i.e., a fast pH-independent route in TMPRSS2+ cells (Fig. 2D, 3F to 3H, and 4D) and a slow acid-activated route in cells lacking TMPRSS2 (Fig. 2C, 3E, 3G, and 3H).

Our view and model are further supported by new gain-of-function experiments with TMPRSS2 that are now shown in Figure 4D. SARS-CoV-2 infections in A549 cells became fully resistant to bafilomycin A1 when TMPRSS2 was expressed, whereas it was completely inhibited in the parental A549* cells that do not express TMPRSS2 (Fig. 4D). This section has been reworded to include the new results, lines 226-289. Please, see our response to the Major comments 1 and 6 for the details of text modifications.

Major comment 8. The dominant negative and constitutively active Rab7a only have a minor inhibitory effect on the graph compared to control (20-30%) while overexpression of WT Rab7a increases fusion.

Author response:

Here, we choose to normalize the values to those of cell populations with the respective lowest expression of Rab7a wt and mutants because it allowed to correlate the level of SARS-CoV-2 infection with the level of Rab7a expression, i.e., we could detect both potential increases and decreases in infection with increasing levels of the wt and mutant molecules, respectively. However, the analysis cannot only rely on the comparison between low and high expressor cells. A firm conclusion is only possible if the effect of the different proteins on infection is compared for similar levels of mutant and wt Rab7a. For example, we found 110% and 140% infection in cells with a medium and high expression of wt Rab7a, respectively, versus 70% and 65% in cells with corresponding levels of the dominant negative Rab7a, i.e., differences about 40% (=110-70/110) and 50% (=140-65/140), respectively.

Normalization of values to those of cells expressing wt Rab7a for each level of expression would have provided a better appreciation of the effect of Rab7a mutants on SARS-CoV-2 infection. However, we would have missed information on the correlation between infection and expression of wt Rab7a because samples with wt Rab7a would have been set to 100 for low, medium, and high expression. It is only the combination of increased and decreased infection with increasing levels of Rab7a wt and mutants, respectively, that supports a role of Rab7a in the infection of TMPRSS2- cells by SARS-CoV-2.

We want to highlight that late endosomal maturation is known to be ineffectively abolished by only targeting Rab7a or microtubule network (Huotari and Helenius, 2011, EMBO J). In general, it results in a relatively low decrease in infection by viruses that require late endosomes for penetration (Khor et al., 2003, Traffic; Quirin et al., 2008, Virology; Lozach et al., 2010, Cell Host Microbe). This is why we complemented our approaches by testing additional perturbants of the late endosome functions (colcemid, MG-132 etc.) and determined the kinetic of acid-dependent penetration. Together these results support our view that SARS-CoV-2 relies on late endosomes for infection of TMPRSS2- cells, but not for infection of TMPRSS2+ cells.

In sum, a 50% difference in infection between the highest expressor cells of Rab7a wt and mutants can confidently be considered as very significant, which was confirmed by robust

statistical analysis. The corresponding section has been reworded to get in clarity, lines 296-315, as follow:

"*TMPRSS2- Vero cells were transfected with DNA plasmids encoding the wild-type (wt), dominant-negative (Rab7a T22N), and constitutively active (Rab7a Q67L) forms of Rab7a tagged with enhanced green fluorescent protein (EGFP) prior to infection with SARS-CoV-2. Transfected cells exhibiting different levels of EGFP expression (low, medium, and high) were selected and then analyzed for infection. Increasing expression of wt Rab7a facilitated SARS-CoV-2 infection. In contrast, increasing the expression of either Rab7a mutant, which perturbates the maturation of newly formed LEs (Lozach, Huotari et al., 2011a, Lozach et al., 2010), resulted in an about 20-30% decrease in infection at the highest expression (Fig. 5A).*

The analysis relies on the assumption that the different Rab7a forms exhibit similar levels of expression, however we found a 1.4-fold higher and a 0.7-fold lower infection in cells with a similar and high expression of wt Rab7a and either mutant of the small GTPase, respectively (Fig. 5A). This comparison revealed a 50% inhibitory effect of the two Rab7a mutants on SARS-CoV-2 infection, indicating that virus fusion is hampered in cells with late endosomal vesicles expressing Rab7a T22N or Q67L. This inhibitory effect was very significant as late endosomal maturation is hard to completely abolish by only targeting Rab7a (Huotari & Helenius, 2011). In general, disruption of LE functions rarely leads to a complete block in infection by viruses relying on LEs for penetration (Khor, McElroy et al., 2003, Lozach et al., 2010, Quirin, Eschli et al., 2008). Taken together, the combination of increased and decreased infection with increasing levels of Rab7a wt and mutants, respectively, suggested that proper maturation of LEs is mandatory for cathepsin L-dependent infectious entry of SARS-CoV-2."

Major comment 9. Cell-cell fusion experiments. The authors should stain the cells (S or N) to show that the syncytia are productively infected Bi-nucleated cells may correspond to cells undergoing division, are many 3+ nuclei cells observed in this assay? There is no evidence of fusion here as there is no marker of cytoplasmic or membrane mixing.

Author response:

We have improved our cell-cell fusion model and used the approach depicted in the drawing in Figure 6D. The new results, including controls, are shown in Figures 6E, 6F, 7C, 7D, and S8 to

S10. Briefly, cells were first infected with SARS-CoV-2 for 24 h and then cocultured for 5 h along with non-infected, target cells that were prestained with CMFDA (green). After fixation, nuclei were stained with Hoechst (blue), and infected cells were subjected to immunofluorescence staining against SARS-CoV-2 nucleoprotein (magenta) prior imaging. In this assay, syncytia appeared in white. This new approach confirmed our initial results. The new cell-cell fusion model and related results are discussed in lines 384-421 and 434-445 as follow:

"*To relate the proteolytic processing of the SARS-CoV-2 spike with the viral fusion mechanisms, we then evaluated the capacity of the virus to mediate cell-cell fusion, i.e., the formation of syncytia (''fusion-from-within'') (Fig. 6D). Similar systems have recently been described for SARS-CoV-2, and previously, for unrelated viruses (Bratt & Gallaher, 1969, Buchrieser et al., 2020, Papa, Mallery et al., 2021). Briefly, cells were first infected with SARS-CoV-2 and then cocultured along with fresh cells, not infected and prestained with the cytosolic green dye CMFDA. CMFDA has the advantage of freely passing through membranes, but once inside the cells, the dye is no longer able to cross the plasma membrane, thus preventing leakage to neighboring cells. Immunofluorescence staining against the viral nucleoprotein allowed for the distinction between infected and target cells. Confocal images clearly showed infected cells in magenta, target cells in green, and the syncytia that result from the fusion of the two in white, i.e., magenta plus green (Fig. 6E and S8 to S10).*

The infection of parental A549 and Vero cells led to the formation of a marginal number of syncytia, and all were small, with only two-three nuclei (Fig. 6E, S9, and S10). In contrast, large syncytia with six or more nuclei were observed upon infection of TMPRSS2+ A549* cells (Fig. 6E and S8). The extent of cell-cell fusion was confirmed with a fusion (f) index that expresses the average number of fusion events per original mononucleated cell (White, Matlin et al., 1981). The (f) index reaches 1 when all the nuclei in the microscope field are present in a single cell, and the value is 0 when all cells have one nucleus each. The (f) index ranged from 0.04 to 0.24 in Vero and A549* cells and reached 0.5 in TMPRSS2+ A549* cells, i.e., a 2- to 13-fold higher compared to that obtained with TMPRSS2- target cells (Fig. 6F). Combined, our results confirmed that the presence of TMPRSS2 on target cells promotes SARS-CoV-2-mediated syncytia formation (Buchrieser et al., 2020), most likely by achieving the proteolysis of the S protein on the surface of infected cells.*

Using our cell-cell fusion model, we then wanted to determine if furin and trypsin can also complete the proteolytic processing of the SARS-CoV-2 spike for fusion. A549 and Vero cells are*

both negative for TMPRSS2 and thus represent a convenient model for monitoring proteolytic activation of cell surface spike proteins by exogenous proteases. Trypsin treatment of infected A549 and Vero cells co-cultured with CMFDA+ target cells resulted in the formation of large syncytia with multiple nuclei (Fig. S9 and S10), similar to those observed after infection of TMPRSS2+ A549* cells (Fig. 6E and S8). In contrast, no difference was observed between furin- and mock-treated infected A549* and Vero cells, among which the only cells with more than one nucleus were actively dividing cells (Fig. S9 and S10). Additionally, the fusion index in A549* and Vero cells was significantly increased under trypsin treatment compared to mock- and furin-treated cells (Fig. 6F). Of note, neither furin nor trypsin treatment enhanced syncytia formation upon infection of TMPRSS2+ A549* cells (Fig 6F and S8). Our data collectively indicated that furin is ineffective in completing spike activation and that proteolytic cleavage is both sufficient and necessary for SARS-CoV-2 membrane fusion.''*

''We next investigated the influence of low pH on SARS-CoV-2 fusion in our cell-cell fusion assay. The formation of syncytia and the (f) index did not differ significantly when cells were treated with low-pH or neutral buffers regardless of the presence of TMPRSS2 (Fig. 7C).

Our results support a model in which endosomal acidification is not essential for SARS-CoV-2 membrane fusion, but SARS-CoV-2 infection relies on low pH for cathepsin L-dependent infection in cells lacking TMPRSS2. Therefore, we tested the possibility that acidic pH is required for the activation of the endolysosomal proteases that trigger SARS-CoV-2 fusion. In such a scenario, the S proteins that are already primed by proteases should no longer require low pH for fusion. Indeed, we found that the fusion index was not increased when trypsin treatment was followed by exposure to a decrease in pH of 7.4 to 5 (Fig. 7C and 7D), the latter value being typical of the luminal pH of endolysosomes (Lozach et al., 2011a).''

Peer Referee #2 comments

SARS-CoV-2 infects cells by binding to the host cell receptor ACE2, through its Spike glycoprotein present on the envelope of the viral particle, which triggers virus-host membrane fusion. Previous studies have show that CoV2 can be cleaved by TMPRSS2 at the plasma membrane or cathepsin L in the endosome, in order to facilitate infection. Most of this work has been carried out using inhibitors of these proteases and in the present manuscript Koch et al. take the same approach and reach similar conclusions. Endosomal acidification is known to be important for cathepsin L mediated entry (eg https://pubmed.ncbi.nlm.nih.gov/33465165/), as first shown for SARS-CoV-1 in 2005 (https://pubmed.ncbi.nlm.nih.gov/16081529/). The data by Koch et al. agrees with this previous work.

Author response:

We thank Reviewer 2 for the comments that we believe helped to improve our study. The line numbers in our responses refer to the **Word file** of the revised manuscript, without any track changes.

We agree that previous studies have shown that TMPRSS2 and cathepsin L are important cell factors for SARS-CoV-2 infection. There is however no experimental demonstration that TMPRSS2 cleaves the SARS-CoV-2 spike on viral particles at the plasma membrane and cathepsin L in endosomes, as underlined by Reviewer 3. The literature in this field remains overall unclear. There are many reasons for this. Conclusions are very often drawn for SARS-CoV-2 based on results obtained for MERS-CoV and SARS-CoV-1, but not for SARS-CoV-2 itself. We agree that SARS-CoV-1 is closely related to SARS-CoV-2, but they remain two distinct viruses. In addition, reports on the cell entry mechanisms of SARS-CoV-2 and other CoVs are often performed in only one cell model system and mostly involve the use of surrogate systems rather than authentic infectious viral particles, e.g., ectopic expression of the SARS-CoV-2 protein S, cell-cell fusion models, pseudotyped particles, etc.

Typically, the two works cited by Reviewer 2 were performed with human immunodeficiency virus (HIV) and murine leukemia virus (MLV) pseudotyped with coronavirus protein S. HIV and MLV are retroviruses that assemble and bud at the plasma membrane of infected cells while the assembly and budding of SARS-CoV-2 occur in the ERGIC (Cortese et al., 2020, Cell Host Microbe; Klein et al., 2020, Nat Com). This raises important questions on the proteolytic processing of the SARS-CoV-2 spike when it is overexpressed to be secreted to the

plasma membrane and incorporated into retrovirus particles. Do pseudotype particles perfectly mimic the proteolytic processing of the S protein exiting cells at the surface of SARS-CoV-2 particles assembled in the ERGIC? Is the S1/S2 cleavage site as accessible in one case as in the other? This has huge implication for the identity of the host cell proteases to achieve the proteolytic processing and activation of the coronavirus in target cells.

Our study reaches a higher degree of complexity using authentic infectious SARS-CoV-2 particles and provides a comprehensive view of SARS-CoV-2 entry mechanisms. It reconciles the apparently contradictory observations from recent reports on endosomal acidification and the roles of furin, TMPRSS2, and cathepsin L in the productive entry and fusion process of SARS-CoV-2. Our results revealed the central role of TMPRSS2 in the cell entry of SARS-CoV-2, which can use two different, mutually exclusive routes to enter and infect target cells, i.e., a fast pH-independent route in TMPRSS2+ cells and a slow acid-activated route in cells lacking TMPRSS2 expression.

We also want to highlight that a preprint of our paper was published on bioRxiv in December 2020 (https://www.biorxiv.org/content/10.1101/2020.12.22.423906v1), and our manuscript was already under the reviewing process when papers addressing similar questions were accepted for publication.

Major point 1. The authors claim that the Vero cells are TMPRSS2 negative but some cell-cell fusion events can still be detected when they are infected with SARS-CoV-2. The authors should provide an explanation for this, as the small amount of TMPRSS2 present in Vero cells can influence the comparison with other cell lines in infection experiments in the manuscript.

Author response:

We agree with Reviewer 2 that some cell-cell fusion events can be detected when Vero cells are infected with SARS-CoV-2, but the number of syncytia is minimal and their size small in comparison to the infected Vero cells subjected to trypsin treatment. It could be that cell surface proteases other than TMPRSS2 can process the spike but with lower efficiency. Alternatively, we cannot rule out that a small amount of TMPRSS2 may actually contribute to the spike activation and cell-cell fusion. Such a scenario would thus support the idea that the more protease is expressed in the target cells, the more molecules of spike is cleaved and activated, and the more fusogenic the virus is, which overall fits our model with TMPRSS2 as a key player in driving SARS-CoV-2

entry. This view is further supported by our new data showing cell-cell fusion with either parental A549* cells (TMPRSS2-) or TMPRSS2-overexpressing A549* cells (Fig. 6E and 6F). Parental A549* cell-cell fusion can still be detected, but as with Vero cells, it is minimal in number and size, whereas huge syncytia are formed with TMPRSS2-overexpressing A549* cells. These new results are discussed in lines 396-421 and shown in Figures 6E, 6F, and S8 to S10. Please, see our response to Minor Comment 8 of Reviewer 2 for more details about our improved cell-cell fusion model.

"*The infection of parental A549* and Vero cells led to the formation of a marginal number of syncytia, and all were small, with only two-three nuclei (Fig. 6E, S9, and S10). In contrast, large syncytia with six or more nuclei were observed upon infection of TMPRSS2+ A549* cells (Fig. 6E and S8). The extent of cell-cell fusion was confirmed with a fusion (f) index that expresses the average number of fusion events per original mononucleated cell (White, Matlin et al., 1981). The (f) index reaches 1 when all the nuclei in the microscope field are present in a single cell, and the value is 0 when all cells have one nucleus each. The (f) index ranged from 0.04 to 0.24 in Vero and A549* cells and reached 0.5 in TMPRSS2+ A549* cells, i.e., a 2- to 13-fold higher compared to that obtained with TMPRSS2- target cells (Fig. 6F). Combined, our results confirmed that the presence of TMPRSS2 on target cells promotes SARS-CoV-2-mediated syncytia formation (Buchrieser et al., 2020), most likely by achieving the proteolysis of the S protein on the surface of infected cells.*

Using our cell-cell fusion model, we then wanted to determine if furin and trypsin can also complete the proteolytic processing of the SARS-CoV-2 spike for fusion. A549 and Vero cells are both negative for TMPRSS2 and thus represent a convenient model for monitoring proteolytic activation of cell surface spike proteins by exogenous proteases. Trypsin treatment of infected A549* and Vero cells co-cultured with CMFDA+ target cells resulted in the formation of large syncytia with multiple nuclei (Fig. S9 and S10), similar to those observed after infection of TMPRSS2+ A549* cells (Fig. 6E and S8). In contrast, no difference was observed between furin- and mock-treated infected A549* and Vero cells, among which the only cells with more than one nucleus were actively dividing cells (Fig. S9 and S10). Additionally, the fusion index in A549* and Vero cells was significantly increased under trypsin treatment compared to mock- and furin-treated cells (Fig. 6F). Of note, neither furin nor trypsin treatment enhanced syncytia formation upon infection of TMPRSS2+ A549* cells (Fig 6F and S8). Our data collectively indicated that*

furin is ineffective in completing spike activation and that proteolytic cleavage is both sufficient and necessary for SARS-CoV-2 membrane fusion."

We now discussed the possible correlation between TMPRSS2 expression, proteolytic processing of the SARS-CoV-2 protein S, and viral fusion in lines 561-577 as follow:

"*Our results suggested that the proteolytic activation of the spike S protein was sufficient and necessary for SARS-CoV-2 fusion. The Vero cells used in our virus-mediated cell-cell fusion assay did not express TMPRSS2 on the cell surface, or at least at a detectable level. In this assay, exogenous furin failed to promote syncytia formation, indicating that furin cleavage was either inefficient or not sufficient to achieve the full activation of the S protein at the plasma membrane. The S1/S2 site of SARS-CoV-2 S protein exhibits an RRAR motif instead of the typical RX(R/K)R furin motif, and a recent structural study indicates that cleavage by furin at this site in S trimers is rather low, approximately 30% (Bestle et al., 2020, Cai, Zhang et al., 2020, Wrobel et al., 2020). However, we found that, unlike furin, trypsin prompted the formation of syncytia, which supports the proposed involvement of the proteases within target cells, such as TMPRSS2 and cathepsin L, in completing the proteolytic processing of the S protein. Others have shown that SARS-CoV-2 and MERS-CoV mediate cell-cell fusion at neutral pH without any further proteolytic treatment when target cells express TMPRSS2 (Buchrieser et al., 2020, Shirato, Kawase et al., 2013). It is tempting to postulate that the more protease is expressed in the target cells, the more molecules of spike is cleaved and activated, and the more fusogenic the virus is. Additionally, work evaluating SARS-CoV-2 infection of primary human intestinal epithelial cells showed that TMPRSS2 expression was the best indicator of cell tropism (Triana, Metz-Zumaran et al., 2021).*"

Major point 2. The western blot in Figure 1A which compares the TMPRSS2 levels in cell lines is not convincing. The actin in Caco2 lane is roughly the double that of Vero and A549* and so is TMPRSS2; this raises doubts about the actual amount of TMPRSS2 in those cell lines. The authors should provide a new western blot loading the same amount of cell lysate and show quantification analysis of TMPRSS2 for each cell line.

Author response:

We apologize for the poor quality of the original image. We now show better quality TMPRSS2 western blot images with normalization to EF2 and semiquantification (Fig. 1A). We want to

highlight that endogenous TMPRSS2 is hard to detect with commercial antibodies. We have tested different antibodies and conditions, and we now show blots performed with the antibody ab92323 from Abcam. Using this antibody, we observed two bands for TMPRSS2 in Caco-2 cells (Fig. 1A), a band of approximately 50 kDa, which is the full-length protein, and a second band of approximately 40 kDa, which is believed to be a cleave form of TMPRSS2 (Chen et al., 2010, Am J Pathol). Similar observations were made in A549 cells that express a transgene coding for TMPRSS2 (Fig. 4A). Altogether, these new blots confirmed that TMPRSS2 was not expressed in Vero and A549* cells to a detectable level and was expressed at much higher levels in Calu-3 and Caco-2 cells (Fig. 1A), corroborating results from other groups (Steuten et al., 2021, Zecha, Lee et al., 2020). The new blots are described in lines 115-120 as follow:

"*When cell lysates were subjected to SDS-PAGE and western blotting, we found that TMPRSS2 was effectively expressed in Calu-3 cells and to a lesser extent in Caco-2 cells (Fig. 1A), corroborating results from other groups (Steuten et al., 2021, Zecha, Lee et al., 2020). TMPRSS2 was seen as a band of approximately 50 kDa in both Calu-3 and Caco-2 cells. A second band of approximately 42 kDa was observed in Caco-2 cells, which represents a cleaved form of TMPRSS2 (Chen, Lee et al., 2010).*"

Major point 3. The use of inhibitors to dissect the different ways of entry and fusion is not ideal for this type of study due to off-target activity or incomplete inhibition of the targeted protease, which can alter downstream results. In particular, the Aprotonin used in this study may block not only TMPRSS2 but also other serine proteases (e.g. TMPRSS4) which can be involved in the entry process. Genetic knockout (eg. TMPRSS2 KO) should be used to confirm the results and examine the exact contribution of TMPRSS2 protease in SARS-CoV-2 entry.

Author response:

To address the possible lack of aprotinin specificity, we tested the capacity of camostat mesylate to hamper SARS-CoV-2 infection in our four cell lines. Camostat mesylate is proposed to be more specific and potent in blocking TMPRSS2 than aprotinin. Like aprotinin, camostat mesylate only impaired SARS-CoV-2 infection in TMPRSS2+ cells, i.e., Calu-3 and Caco-2 cells. The new results are shown in Figure S3 and described in lines 160-162 as follow:

"*Similar results were obtained with camostat mesylate (Fig. S3), a more specific and potent inhibitor of TMPRSS2 than aprotinin, also known to block SARS-CoV-2 infection (Hoffmann et al., 2020b).*"

Additionally, we added a new figure (Fig. 4) that shows results obtained with A549* cells overexpressing TMPRSS2 (Fig. 4A). Briefly, camostat mesylate blocked SARS-CoV-2 infection in TMPRSS2+ A549* cells in a dose-dependent manner, but not at all in parental A549* cells (Fig. 4B). Conversely, cathepsin L inhibitor (SB412515) impaired infection only in parental cells, and not in TMPRSS2+ A549* cells (Fig. 4C). Additionally, SARS-CoV-2 infections in A549* cells became resistant to agents that elevate endosomal pH when TMPRSS2 was expressed (Fig. 4D). Together, these new results support our model that SARS-CoV-2 can use distinct, mutually exclusive entry routes and that TMPRSS2 is important for sorting the virus into either pathway. The results are discussed in lines 271-289 as follow:

"*To correlate the presence of TMPRSS2 with the viral entry pathway, we examined SARS-CoV-2 infection in the same cell line expressing or lacking the protease. To this end, we stably expressed TMPRSS2 in the TMPRSS2- A549* cells and confirmed the overexpression by SDS-PAGE and western blotting (Fig. 4A) (Steuten et al., 2021). As expected, the TMPRSS2 inhibitor, camostat mesylate, did not inhibit SARS-CoV-2 infection when it was added to the parental A549* cells prior to infection (Fig. 4B). However, we found that camostat mesylate reduced SARS-CoV-2 infection in a dose-dependent manner in TMPRSS2-overexpressing A549* cells (Fig. 4B). Conversely, the cathepsin L inhibitor (SB412515), efficiently prevented infection of regular A549* cells but had no effect on infection of A549* cells that expressed TMPRSS2 (Fig. 4C).*

Our results indicated that endosomal acidification is required for SARS-CoV-2 infection of TMPRSS2- cells but not for infection of cells expressing TMPRSS2 (Fig. 3E to 3H). To evaluate the role of TMPRSS2 in the dependence of SARS-CoV-2 on low pH for entry, A549 cells expressing or lacking TMPRSS2 were infected in the presence of bafilomycin A1 to neutralize endosomal pH. As anticipated, infection of TMPRSS- A549* cells with SARS-CoV-2 decreased dramatically with increasing concentrations of bafilomycin A1 (Fig. 4D). In contrast there was no noticeable effect of bafilomycin A1 on infection of TMPRSS2+ A549* cells. This confirmed that SARS-CoV-2 infection does not rely on endosomal acidification when TMPRSS2 is expressed. Overall, TMPRSS2 appears as the major determinant of the fast pH-independent route taken by SARS-CoV-2 to enter and infect TMPRSS2+ cells.*"

Major point 4. It is concerning that different MOIs are used for the different cell lines, and are not consistent between different experiments (Fig.1C and 1D). Furthermore, different inhibitors concentrations were used for different cell lines. It is important to be consistent throughout the assays or clarify in the text why different MOIs and drug concentrations were used and how they were determined (see minor comments for details).

Author response:

Different MOIs were used in each experiment as the MOI was determined on Vero cells and not on each cell line. We used different MOIs for each cell line with the aim of achieving about 20% infection, which generally avoids saturation and allows us to detect either an inhibitor or enhancer effect of perturbants. That 20% of infection was not reached with the same MOI throughout the assays for each cell line simply reflects the difference in sensitivity of our four cell lines to SARS-CoV-2 infection. SARS-CoV-2 was produced from Vero cells, and supernatants were previously titered on Vero cells by TCID50 assay. We agree that it was insufficiently explained in the original manuscript, and furthermore, that TCID50 values cannot be used strictly speaking to define the MOI. To clarify this important point, the MOI for SARS-CoV-2 is now given according to the titer determined on Vero cells by plaque-forming unit assay, and the definition of MOI is described in the MM section, lines 644-650, as follow:

"*For titration of SARS-CoV-2, confluent monolayers of Vero cells were infected with 10-fold dilutions of virus in serum-free medium and then grown in the presence of complete medium containing 2% serum and 0.05% agarose to prevent virus spread. Plaques were stained by crystal violet three days post-infection. The MOI was assigned for SARS-CoV-2 according to the titer determined on Vero cells. The MOI for SFV and UUKV was given based on the titers determined on BHK-21 cells as previously described (Lozach et al., 2010).*"

We also indicated the MOI used for each cell line and experiments in the figure legends and clarified why different MOIs were used by rewording the last paragraph in the first section of the result part in lines 148-152 as follow:

"*In all further experiments, as we aimed to characterize SARS-CoV-2 entry mechanisms, we limited our assays to 8 hpi. In addition, as our cell lines differed in their sensitivity to SARS-CoV-2 infection, we used different MOIs for each cell line allowing the infection of approximately 20%*

of the cells. This range of infection generally avoids saturation of cells and thus allows detection of potential inhibitory or enhancing effects of a perturbant."

For the inhibitors, we followed a standard biophysics procedure to determine the IC_{50} value of each drug on each cell line. As the sensitivity of SARS-CoV-2 infection to many of these drugs greatly differs depending on whether TMPRSS2 is expressed or not, this led to the use of a wide range of drug concentrations.

Major point 5. The experiments exploring the role of furin in processing and activating Spike protein and its role in cell-cell fusion are also concerning. In figure 6, the virus was produced in cells expressing furin. The effect of furin cannot be truly quantified as all the newly produced particles already contain a pre-cleaved Spike at the S1-S2 site. The same issue is true for the contribution of furin cleavage to the cell-cell fusion. Most of the Spike protein produced by infected cells and exposed on the cell surface to mediated cell-cell fusion is already cleaved, so adding furin to the medium may not have any detectable effect, leading to a misleading conclusion. This experiment should be run in Vero cells knocked out for furin and then adding the furin enzyme to the medium after infection.

Author response:

In our study, SARS-CoV-2 was indeed produced from Vero cells that express furin. From the literature, it appears that not all the newly produced particles already contain a pre-cleaved spike at the S1-S2 site, but only one third (Wrobel et al., 2020, Nat Struct Mol Biol; Tang et al., 2021, ACS Infect Dis). It is exactly what we also observed in our system when SARS-CoV-2 was produced from our Vero cells in the presence of 2% serum. The new results are now shown in Figures 6A and 6B. Our new results also show that adding exogenous furin to virus stock did not significantly promote the proteolytic processing of the S protein. Conversely, upon treatment with trypsin, 92% of the S protein on SARS-CoV-2 particles was cleaved, although the presence of 2% serum in the virus stocks. As the proteolytic processing by furin is apparently inefficient by default, we do not consider it necessary to generate Vero cells knocked-out for this protease. The new set of data regarding the proteolytic cleavage of the S protein are discussed in lines 347-374 as follow:

"*Furin expression in the producer cells and TMPRSS2 expression at the surface of target cells are believed to mediate SARS-CoV-2 activation through proteolytic processing of the S protein. Hence,*

we first evaluated the cleaved spike content in viral particles after biosynthesis. Authentic infectious viruses were analyzed by SDS-PAGE and western blot using a primary antibody recognizing the S2 segment (S2) of the SARS-CoV-2 spike. A strong band corresponding to the full-length SARS-CoV-2 spike, which includes S1 and S2 segments (S0), was detected at 160 kDa (Fig. 6A). A second band, although of lower intensity, was clearly visible at 75 kDa, which corresponds to S2. With a lower extent, additional bands were discernible above 160 kDa, which likely correspond to dimeric and trimeric forms of the SARS-CoV-2 spike. Upon semi-quantifying the intensities of the S0 and S2 bands, the ratio of cleaved spike (S2) to the total of spike, i.e., uncleaved plus cleaved (S0+S2), was found to be one third [(□) = S2/S0+S2] (Fig. 6B). These results were consistent with prediction models based on the SARS-CoV-2 spike structure (Wrobel, Benton et al., 2020).

Next, we assessed the efficiency of exogenous furin and trypsin in processing S0 into S2 on viral particles. In this assay, trypsin was used to mimic TMPRSS2 at the cell surface as the two enzymes are closely related and both belong to the group of trypsin-like proteases. The use of exogenous cathepsin L was excluded because the enzyme is active only at pH ~5, which would have made it impossible to distinguish the effects of low pH from those of proteolytic cleavage. After treatments with the exogenous proteases, samples were analyzed by western blot using the primary antibody against S2. Although the presence of serum in our virus preparations, both trypsin and furin proteolytically processed the full-length form of spike S0 into S2, but with a striking difference in efficiency (Fig. 6A). Trypsin treatment greatly reduced the relative intensity of S0, which resulted in an increase in the intensity of S2, with a (□) value reaching almost 1.0, while furin treatment had a more modest impact as the (□) value increased from 0.3 to 0.5 (Fig. 6B). To confirm that the presence of serum had no impact on protease cleavage of spike, A549 cells expressing or lacking TMPRSS2 were infected with SARS-CoV-2 produced in the presence or absence of serum. Results showed that the presence of serum did not impact SARS-CoV-2 infection and cleavage (Fig. S7).*"

Minor comment 1. Line 116: "The susceptibility of Caco-2 and Vero cells to SARS-CoV-2 at multiplicities of infection (MOIs) of 0.1 and 0.5" Why different MOIs were used for the two cell lines? How was this assessed?

Author response:

Please see above Reviewer 2 Major point 4.

Minor comment 2. Line 119: "Similarly, 35% of Vero cells were infected at 8 hpi (Fig. 1C)." As the number of Vero cells showed in the panel is lower than in Caco2, at least two fields for each cell line with the same number of cells is needed. Including quantification analysis would be beneficial.

Author response:

We thank Reviewer 2 for pointing on this problem in the layout of Figure 1. The values corresponded to the average of many fields. We now show two fields for each line infected with SARS-CoV-2 that illustrate the values obtained from image analysis. We do not consider necessary to have a specific graph showing the image-based quantification of infection in Caco-2 and Vero cells as flow cytometry dot plots are already shown for these cells in Figure 1D. Flow cytometry is much more quantitative, from our opinion. We therefore prefer to keep providing the quantification of microscopy images in the text as follow, lines 133-134:

"*The results showed that 10% of Caco-2 cells were positive for NP while 35% of Vero cells were infected (Fig. 1C).*"

Minor comment 3. Why the MOI used in Fig.1C is 0.1 for Caco2 and 0.5 for Vero cells, while for Fig.1D the difference in MOI is 0.3 for Caco2 and 0.003 for Vero cells? And why the percentage of infected Vero cells in D is only slightly higher than in C although the MOI used in D is around 100 times less?

Author response:

Please see above Reviewer 2 Major point 4. In addition, the infection in Figure 1C was stopped after 8 h, whereas the infection in Figure 1D lasted overnight. This is now more explicitly mentioned in the results, lines 130-134, and in the figure legends.

"*The susceptibility of Caco-2 and Vero cells to SARS-CoV-2 at a multiplicity of infection (MOI) of 0.2 was assessed 8 hours postinfection (hpi) by fluorescence microscopy after immunostaining with a mouse monoclonal antibody (mAb) against the intracellular viral nucleoprotein NP (Fig. 1C). The results showed that 10% of Caco-2 cells were positive for NP while 35% of Vero cells were infected (Fig. 1C).*"

Minor comment 4. Line 205: "NH4Cl disrupts TMPRSS2+ cell-specific functions that are important for SARS-CoV-2 replication". The concentration of NH4CL used may also impact on the replication step in these cell lines. Can the authors perform a NH4CL titration to find the right concentration where entry is inhibited but replication is not affected? Also, the replication is not compromised in Fig.3A where Caco2 showed around 20 % of infected cells in the presence of the NH4Cl. How the authors explain this difference in the two assays?

Author response:

To address the first point, we performed the reverse approach of adding NH_4Cl. This assay consists of washing NH_4Cl out at different times after synchronization and the onset of infection. Results are shown in Figure S6B. They clearly indicate that NH_4Cl had no noticeable effect on SARS-CoV-2 infection in these TMPRSS2+ cells for the first hour after warming, at a time when the virus had already penetrated the cells, but viral replication had not yet begun (Fig. 1F and 2D). In contrast, infection in A549* and Vero cells (TMPRSS2-) decreased during the first 30 min until it was no longer detectable after 2 h (Fig. S6B), indicating overall that SARS-CoV-2 cannot remain

infectious for very long before acid-activated penetration into TMPRSS2- cells. The set of new results is discussed in lines 226-269 as follow:

"*To examine how long SARS-CoV-2 remains acid-activable in TMPRSS2- cells, we followed the reverse approach of adding NH4Cl. This assay relies on the fact that the neutralization of endosomal pH by NH4Cl is reversible after washing. Virus binding to Vero cells was synchronized at low MOI (~0.3) on ice, and cells were rapidly shifted to 37°C in the presence of NH4Cl before the weak base was washed out at varying times. In other words, we determined the time at which SARS-CoV-2 acid activation was no longer possible. In Vero cells, infection decreased by 70% during the first 30 min and then more slowly until it reached a 90% decrease after 2 h (Fig. S6B), which was the exact opposite of the NH4Cl addition approach (Fig. 3E). Together, the NH4Cl add-in and wash-out kinetics indicated that SARS-CoV-2 infectivity decreases sharply in TMPRSS2- cells if the virus is not allowed to enter the cytosol rapidly by acid-activated penetration, most likely, from endosomal vesicles.*

In Calu-3 and Caco-2 cells, both of which express TMPRSS2, it was not possible to determine the timing of the acid-requiring step. We failed to detect SARS-CoV-2-infected cells when NH4Cl was added, even if the addition occurred several hours after transferring the cells from 4 to 37°C (Fig. 3E). In samples where NH4Cl was omitted, however, infection was readily detectable with 17% of Calu-3 and Caco-2 cells infected (Fig. S6A) suggesting that the weak base interferes with SARS-CoV-2 replication in these two cell lines. To clarify whether NH4Cl blocks virus entry or replication in these cells, we next assessed SARS-CoV-2 infection in our wash-out assay. Neutralization of endosomal pH with NH4Cl in Caco-2 cells had no noticeable effect on SARS-CoV-2 infection for the first hour after warming (Fig. S6B), when the virus had normally already penetrated TMPRSS2+ cells (Fig. 2D). In this assay, viral replication was not affected, most likely because the weak base was washed out early enough to avoid side effects. Altogether, these results suggest that SARS-CoV-2 does not depend on endosomal acidification for infectious entry into TMPRSS2+ cells, but rather that NH4Cl disrupts Calu-3 and Caco-2 cell-specific functions that are important for SARS-CoV-2 replication. NH4Cl not only neutralizes the intracellular pH but also alters all endosomal, lysosomal, and trans-Golgi network functions that are acid dependent (Helenius, 2013).

As an alternative method to alter endosomal pH, we added the vATPase inhibitor concanamycin B to cell-bound virus, instead of NH4Cl, at different times after warming. The time course showed that infectious entry of UUKV began later than 15 min and had not reached a

maximum 2 h after cell warming. In marked contrast, SARS-CoV-2 infection was virtually insensitive to the concanamycin B addition as early as a few seconds after shifting TMPRSS2+ cells to 37°C (Fig. 3F). As expected, SARS-CoV-2 passed the concanamycin B-sensitive step in TMPRSS2- cells within less than 15 min, and infectious entry reached a plateau value after 45 min, somewhat faster than in cells treated with NH4Cl (Fig. 3G). This difference in sensitivity to endosomal pH may be because concanamycin B interferes not only with endosomal functions that are acid dependent but also, indirectly, with the maturation of endosomes. However, unlike NH4Cl, it was apparent that concanamycin B had no adverse effect on SARS-CoV-2 replication in any of these experiments. Neutralization of endosomal pH by chloroquine lead to similar kinetics of SARS-CoV-2 infection as concanamycin B (Fig. 3H).

Taken together, these results strongly suggest that SARS-CoV-2 can use two different routes to enter and infect target cells, i.e., a fast pH-independent route in TMPRSS2+ cells (Fig. 2D, 3F, S6B, and 3H) and a slow acid-activated route in cells lacking TMPRSS2 (Fig. 2C, 3E, 3G, and 3H)."

Our new results also clearly show that SARS-CoV-2 infection does not rely on low pH and endosomal acidification in TMPRSS2-expressing cells (Figure 4D). SARS-CoV-2 infections in A549 cells became fully resistant to bafilomycin A1 when TMPRSS2 was expressed, whereas it was completely inhibited in the parental A549* cells that do not express TMPRSS2 (Fig. 4D). The new results are described in lines 280-289.

"*Our results indicated that endosomal acidification is required for SARS-CoV-2 infection of TMPRSS2- cells but not for infection of cells expressing TMPRSS2 (Fig. 3E to 3H). To evaluate the role of TMPRSS2 in the dependence of SARS-CoV-2 on low pH for entry, A549* cells expressing or lacking TMPRSS2 were infected in the presence of bafilomycin A1 to neutralize endosomal pH. As anticipated, infection of TMPRSS- A549* cells with SARS-CoV-2 decreased dramatically with increasing concentrations of bafilomycin A1 (Fig. 4D). In contrast there was no noticeable effect of bafilomycin A1 on infection of TMPRSS2+ A549* cells. This confirmed that SARS-CoV-2 infection does not rely on endosomal acidification when TMPRSS2 is expressed. Overall, TMPRSS2 appears as the major determinant of the fast pH-independent route taken by SARS-CoV-2 to enter and infect TMPRSS2+ cells.*"

Together our results indicate that NH_4Cl does not inhibit SARS-CoV-2 infection by elevating endosomal pH and blocking virus entry, but rather by disrupting Calu-3 and Caco-2 cell-

specific functions that are important for SARS-CoV-2 replication. NH_4Cl not only neutralizes the intracellular pH but also alters all endosomal, lysosomal, and trans-Golgi network functions that are acid dependent. As an illustration, the IC_{50} value determined for NH_4Cl in Caco-2 cells was about 8 mM (Table 1), i.e., 4-fold higher than the IC_{50} found in TMPRSS2- cells (about 2 mM). In this scenario, the inhibitor effect of NH_4Cl in Calu-3 and Caco-2 cells, even if it is indirect, is also dose dependent. As noticed by Reviewer 2 in the Minor Comment 12, 75 mM of NH_4Cl was used in the add-in time course assay (Fig. 3E), versus 33 mM in Fig. 3A where about 20% of Caco-2 cells remained infected in the presence of the weak base.

Minor comment 5. Have the authors considered to test Chloroquine rather than NH4Cl (see https://pubmed.ncbi.nlm.nih.gov/33465165/)?

Author response:

Results for chloroquine were shown in Figure 3B of the original submission. We believe that the reviewer meant that we should use chloroquine instead of NH_4Cl in the add-in assays shown in Figure 3E. Of note, it was not possible to completely inhibit infection with chloroquine in Calu-3 and Caco-2 cells (TMPRSS2+) in a range of concentrations that was not cytotoxic to the cells, i.e., > 100 µM (data not shown), consistent with the results from Ou et al. To follow the reviewer's suggestion, we now show the kinetic of penetration in our four cell lines as assessed with chloroquine (Fig. 3H), which perfectly mirrored the results obtained with concanamycin B (Fig. 3G). Altogether, our results indicate that the blockade of SARS-CoV-2 infection by NH_4Cl and Chloroquine in Calu-3 and Caco-2 cells (TMPRSS2+) is not directly due to the neutralization of endosomal pH. The new results are discussed in lines 264-269 as follow:

"*Neutralization of endosomal pH by chloroquine lead to similar kinetics of SARS-CoV-2 infection as concanamycin B (Fig. 3H).*

Taken together, these results strongly suggest that SARS-CoV-2 can use two different routes to enter and infect target cells, i.e., a fast pH-independent route in TMPRSS2+ cells (Fig. 2D, 3F, S6B, and 3H) and a slow acid-activated route in cells lacking TMPRSS2 (Fig. 2C, 3E, 3G, and 3H)."

Minor comment 6. Line 203: "detectable with 17% of Calu-3 and Caco-2 cells infected (data not shown)". Please include these data to allow a comparison.

Author response:

These data are now shown in Figure S6A and cited in the text, lines 220-222, as follow:

"*To ensure that viral penetration is completely abolished after adding NH4Cl, a concentration of up to 75 mM was used (Fig. S6A).*"

Minor comment 7. Line 281: "as described for unrelated viruses". The authors should mention that SARS-CoV-2 Spike protein was already demonstrated to trigger cell to cell fusion and provide references of the studies.

Author response:

The text has been reworded and now reads in lines 384-388 as follow:

"*To relate the proteolytic processing of the SARS-CoV-2 spike with the viral fusion mechanisms, we then evaluated the capacity of the virus to mediate cell-cell fusion, i.e., the formation of syncytia (''fusion-from-within'') (Fig. 6D). Similar systems have recently been described for SARS-CoV-2, and previously, for unrelated viruses (Bratt & Gallaher, 1969, Buchrieser et al., 2020, Papa, Mallery et al., 2021).*"

Minor comment 8. The fusion assay used in Fig.5 to look at cell-cell fusion in different pH conditions should be improved for clarity. Since the detection of a small number of fusion events (in Vero cells) may be rather difficult in this assay, have the authors considered establishing a dual color assay or a fluorescent protein-based assay (e.g. Split-GFP based system)? This would allow a more precise quantification of the fusion events and the impact of pH in Spike mediated cell-cell fusion process.

Author response:

We have improved our cell-cell fusion model and used the approach depicted in the drawing in Figure 6D. The new results, including controls, are shown in Figures 6E, 6F, 7C, 7D, and S8 to

S10. Briefly, cells were first infected with SARS-CoV-2 for 24 h and then cocultured for 5 h along with non-infected, target cells that were pre-stained with CMFDA (green). After fixation, nuclei were stained with Hoechst (blue), and infected cells were subjected to immunofluorescence staining against SARS-CoV-2 nucleoprotein (magenta) prior imaging. In this assay, syncytia appeared in white. This new approach confirmed our initial results. The new cell-cell fusion model is presented in lines 384-395 as follow:

"*To relate the proteolytic processing of the SARS-CoV-2 spike with the viral fusion mechanisms, we then evaluated the capacity of the virus to mediate cell-cell fusion, i.e., the formation of syncytia (''fusion-from-within'') (Fig. 6D). Similar systems have recently been described for SARS-CoV-2, and previously, for unrelated viruses (Bratt & Gallaher, 1969, Buchrieser et al., 2020, Papa, Mallery et al., 2021). Briefly, cells were first infected with SARS-CoV-2 and then cocultured along with fresh cells, not infected and prestained with the cytosolic green dye CMFDA. CMFDA has the advantage of freely passing through membranes, but once inside the cells, the dye is no longer able to cross the plasma membrane, thus preventing leakage to neighboring cells. Immunofluorescence staining against the viral nucleoprotein allowed for the distinction between infected and target cells. Confocal images clearly showed infected cells in magenta, target cells in green, and the syncytia that result from the fusion of the two in white, i.e., magenta plus green (Fig. 6E and S8 to S10).*"

Minor comment 9. Line 433: "...trimers cut by furin." Recent study showed that furin is not the only enzyme processing the spike at the S1-S2 site. This should be stated, and references should be provided.

Author response:

This point is now discussed under the light of our new results and recently published articles in lines 580-591 as follow:

"*The list of the involved host cell proteases is most likely not restricted to furin, TMPRSS2, and cathepsin L, as suggested by recent biochemical studies (Jaimes, Andre et al., 2020, Tang et al., 2021). S proteolytic activation might involve the cleavage of sites other than S1/S2 and S2', as was found for the MERS-CoV S protein (Kleine-Weber et al., 2018). Consistent with the results from others, our own data indicated that the cleavage to S1 and S2 is incomplete on SARS-CoV-2*

particles, most likely with only one of the three S1/S2 sites within S trimers cut by furin in producer cells. As recently proposed, cutting at all the S1/S2 sites would be achieved in this model with the aid of proteases within target cells, such as TMPRSS2 and cathepsin L (Ou, Mou et al., 2021, Tang et al., 2021). The fusogenic conformational change would then occur and be completed by the cleavage of the S2' sites to unmask the fusogenic units. The amino acid sequence of the S1/S2 site differs significantly among CoVs (Bestle et al., 2020), and it is highly likely that the sequence influences the overall viral fusion process."

Minor comment 10. Figure 1E: Can the authors explain why they chose to use MFI rather than the same quantification assay as in D (% of NP positive cells)? It would be better to be consistent through the experiments.

Author response:

As our study is about characterizing SARS-CoV-2 entry, our strategy in flow cytometry-based infection assays was (i) to limit our analysis to incoming viruses, and hence avoid subsequent rounds of infection, (ii) while keeping the synthesis of newly viral nucleoprotein high enough to be detected in our assays, i.e., this was our readout for measuring productive entry. To reach this objective, we determined the timing of viral replication and production of infectious progeny in our cell lines (Fig. 1E to 1G). In the flow cytometry assays, the percentage of infection reflects how many cells are infected and the MFI how much protein there are expressing. The latter parameter is more appropriate to measure and determine the shortest possible timing to detect the viral replication, while the percentage value more accurate when the replication and associated fluorescence signal is minimal. Therefore, we used MFI only once in Fig. 1E and percentage in all further flow cytometry-based infection assays. We prefer to keep it this way. However, we now show the results in Figure 1E with the percentage of infection instead MFI in Figure S1.

Minor comment 11. Figure 2A: The use of Aprotonin may block other serine proteases (TMPRSS4, TMPRSS11D, TMPRSSD11E) which have been showed to play a role in SARS-CoV-2 S priming (https://doi.org/10.1038/s41598-020-78402-2). A genetic knockout of the TMPRSS2 protease in TMPRSS2+ Caco2 and Calu3 cells is needed to dissect the exact role of TMPRSS2 protease in SARS-CoV-2 entry.

Author response:

Please, see our response to Reviewer 2 Major Point 3.

Minor comment 12. Figure 3E: NH4Cl concentration used is 50mM for A549 and Vero and 75mM Calu-3 Caco-2. Why there is this difference compared to the concentrations tested in Fig 3A? It would be better to be consistent through the assays.

Author response:

It is not possible to be consistent with the drug concentrations through the assays because SARS-CoV-2 infection greatly differs in its sensitivity to the different drugs depending on whether TMPRSS2 is expressed or not. Infection in the presence of 33 mM NH_4Cl was still readily detectable in three of our four cell lines (Fig. 3A). To accurately determine the half time of acid-dependent viral penetration (Fig. 3E), it is essential to ensure that further penetration is prevented once the weak base is added. Therefore, we used the maximal NH_4Cl concentration in each cell line for which we obtained a complete abolishment of SARS-CoV-2 infection. The inhibition with 50 and 75 mM NH_4Cl is now shown in Figure S6A. Of note, it is commonly accepted that up to 50-100 mM NH_4Cl is not detrimental for cells, which was confirmed, at least, in our four cell lines with our cytotoxicity assay (Fig. S5). This point is now clarified in text, lines 220-222, as follow:

"*To ensure that viral penetration is completely abolished after adding NH4Cl, a concentration of up to 75 mM was used (Fig. S6A).*"

Minor comment 13. Figure 3F: Why Calu3 and A549* were omitted from this graph? Please include them.

Author response:

Calu-3 and A549* cells are now included in the graph showing add-in time courses with concanamycin B (Figures 3F and 3G, respectively).

Minor comment 14. Figure 4F: Why different concentrations of MG132 were used between Calu3 and Caco2? This may be confusing; it would be clearer to show the same concentration range for both cell lines. How the authors explain the different sensitivity to MG132 between Caco2 and Calu3 at 3.7 uM? Please clarify.

Author response:

We now show the same range of MG-132 concentrations for both Calu-3 and Caco-2 cells (Figure 5F). We believe that the effect of MG-132 on SARS-CoV-2 infection in Calu-3 and Caco-2 cells is not due to the impairment of the late endosomal maturation but is rather indirect. This conclusion is further supported by new data showing that SARS-CoV-2 infection in A549* cells became resistant to MG-132 when TMPRSS2 was expressed (Fig. 5H). This is now further discussed in lines 336-345 as follow:

"*Inhibitors of LE maturation, i.e., colcemid and MG-132, also reduced SARS-CoV-2 infection in a dose-dependent manner in TMPRSS2+ cells (Calu-3 and Caco-2 cells) (Fig. 5E and 5F). Although the inhibition was significant, the decrease in infection caused by colcemid was barely 25-30% (Fig. 5E). The IC50 values of MG-132 were one to three logs higher in TMPRSS2+ cells than in TMPRSS2- cells (Fig. 5F and Table 1). As shown in Fig. 5G, infection was not readily detectable in Calu-3 and Caco-2 cells when MG-132 was added at 2 hpi. Additionally, MG-132 had no effect on SARS-CoV-2 infection of TMPRSS2-overexpressing A549* cells (Fig. 5H). This result contrasted with the infection of parental A549* cells, which decreased with increasing amounts of drug (Fig. 5H). Taken together, these data suggested that the TMPRSS2-dependent SARS-CoV-2 entry pathway does not rely on LE maturation.*"

Minor comment 15. Figure 4G: Please clarify why 60µM MG132 was used in this experiment instead of using the concentrations tested in 4F.

Author response:

Please, see our response to Reviewer #2 Minor Comment 4. Briefly, a concentration of 33 µM MG-132 was not sufficient to completely abolished SARS-CoV-2 infection in Caco-2 cells (Fig. 5F). Therefore, we used a higher concentration of MG-132 in the add-in assays with Caco-2 cells shown in Figure 5G to reach a higher block in infection (Fig. 5G) but no cytotoxicity to the cells (Fig. S5), i.e., 60 µM. In this assay, we concluded to an entry-independent effect of MG-132 in Calu-3 and Caco-2 cells (TMPRSS2+), which is now further documented with the results shown in Figure 5H (for more details, please, see our response to Minor comment 14).

Minor comment 16. Figure 5C: Please provide data using viral protein staining (Nucleoprotein or Spike) to prove that the fused cells are infected. Why does cell-cell fusion occur in the absence of TMPRSS2? The fact that cell-cell fusion is directly correlated to TMPRSS2-mediated cleavage of Spike and is still detected in Vero cells in absence of trypsin suggests that certain levels of TMPRSS2 are still present in this cell line, which will also influence infection data in the study.

Author response:

The staining of intracellular SARS-CoV-2 nucleoprotein is now shown in Figure 6E and S8 to S10. Please, read our answer to the Minor Comment 8 for more details, and for the concern about TMPRSS2 expression, our response to Major Point 1.

Minor comment 17. Figure 6A: Was this virus produced in presence or absence of serum (It is not specified in M&M section)? If the virus was amplified and resuspended in presence of serum, this may inactivate part of the trypsin added, underestimating the infection data.

Author response:

The virus was produced in the presence of 2% serum, which is now specifies in the MM section, lines 641-642, as follow:

"*The virus was amplified in Vero cells in the presence of 2% serum, and working stocks were used after three passages.*"

We do not believe that trypsin was inactivated in our assay and infection underestimated for the reasons exposed in our response to Major Comment 5. Briefly, the proteolytic processing of the S protein was incomplete on viral particles, and S could be almost entirely processed by adding exogenous trypsin although the presence of 2% serum (Fig. 6A and 6B). In addition, we now show additional results showing that SARS-CoV-2 follows the same entry pathways regardless of the presence or absence of serum in virus stocks. The new results are shown in Figure S7 and discussed in lines 370-374 as follow:

"*To confirm that the presence of serum had no impact on protease cleavage of spike, A549* cells expressing or lacking TMPRSS2 were infected with SARS-CoV-2 produced in the presence or absence of serum. Results showed that the presence of serum did not impact SARS-CoV-2 infection and cleavage (Fig. S7).*"

Minor comment 18. Figure 6A-B: Was the virus produced in cells expressing furin? If so, the effect of furin cannot be quantified as most of the particle produced have an already pre-cleaved Spike. A western blot to show the S cleavage on virions would give the exact amount of cleaved spike present before and after furin treatment.

Author response:

Please, see our response to Reviewer 2 Major Point 5.

Minor comment 19. Figure 6B: Please provide a staining with a viral protein to detect the infected cells.

Author response:

The staining of intracellular SARS-CoV-2 nucleoprotein is now shown in Figures 6E and S8 to S10. For more details please read our responses to Major Point 1 and Minor Comments 8 and 16.

Minor comment 20. Figure 6C: See major comment on furin (last point)

Author response:

Please, see our response to Major Point 5.

Peer Referee #3 comments

Koch et al. ('Host Cell Proteases Drive 1 Early or Late SARS-CoV-2 Penetration') set out to determine if SARS-CoV-2 enters epithelial cells from intracellular compartments or the plasma membrane, and how acidic pH contributes to the early steps of infection. They use different epithelial cell lines commonly used in SARS-CoV-2 research (Vero, A549, Calu-3, Caco-2), either expressing or not expressing the SARS-CoV-2 activating trypsin-like protease TMPRSS2. They confirm that TMPRSS2 is an essential host factor for SARS-CoV-2 infection. Evidence is largely based on chemical inhibitors, such as aprotinin, SB142515, MG132, and a range of acidification inhibitors.

The study is, unfortunately, somewhat shallow and incomplete. It remains descriptive, and does not elucidate what function these putative entry pathways would have for SARS-CoV-2. It is unclear if the virus penetrates from the plasma membrane or an early endosomal vesicle in TMPRSS2 positive cells. Cell biological correlate analyses of trafficking or drug controls are largely lacking. This is a pity as it might lead the authors to discover a mechanism, for example towards the question what prevents the virus from being endocytosed in cells expressing the activating protease TMPRSS2.

We thank the reviewer for their time and critical points to help us improve our manuscript. The line numbers in our responses refer to the **Word file** of the revised manuscript, without any track changes.

Major point 1. The title is misleading as it suggests that SARS-CoV-2 uses early endosomes for infection. The manuscript does not furnish unequivoval evidence for this claim. In TMPRSS2 expressing cells, plasma membrane fusion cannot be excluded.

Author response:

We agree with the reviewer that "early penetration" might be misleading. To take into consideration that our data indicate that SARS-CoV-2 can use two distinct entry pathways, i.e., one fast and one slower, we have changed the title as follow:

"*Host Cell Proteases Drive Fast or Slow Entry of SARS-CoV-2 into Cells*".

Major point 2. Authors use inhibitor profiling experiments across different cell lines, for example aprotinin, SB412515, NH4Cl, BafA1, colcemid. I find it a bit daring to compare different cell lines across inhibitor applications, and then try to conclude on a mechanism of action considering whether or not the different cell lines express the TMPRSS2 protease. To me a safer approach would be to prepare TMPRSS2 KO or gain of function cells, and compare their drug susceptibilities to the parental cells.

Author response:

This point is also a concern of Referees 1 and 2. Please, see our responses to the Major Comment 1 of Referee 1 and Major Point 3 of Referee 2.

Major point 3. It is curious that NH4Cl inhibited SARS-CoV-2 infection of Calu-3 and Caco-2 cells irrespective of the time of addition. Authors speculate that this is due to acidification of a compartment used in virus egress. Can they be more specific here, and support this speculation with experimentation? Did they do wash out experiments of NH4Cl?

Author response:

We are not sure what the reviewer means here because we did not discuss virus egress at all in our manuscript. However, we performed wash out experiments of NH_4Cl with Calu-3 and Caco-2 cells while addressing an issue raised by Referee 1. The results are shown in Figure S6B. Please, see our response to the Major Comment 6 of Referee 1 for details.

Major point 4. They use Rab7a overexpression in transfected Vero cells to test in a late step in endosome maturation was involved in SARS-CoV-2 infection. Mutant Rab7a (active or inactive) reduced infection by about 20%. Such transfections have systemic effects on cells, besides the endo-lysosomal pathway. A minor reduction of infection as reported here could easily be due to noise in the assay. These data do not convincingly support the importance of an early to endosome trafficking step in SARS-CoV-2 infection.

Author response:

Thanks for this comment, this was also a concern raised by Reviewer 2. Please, read our response to the Major Comment 8 of Referee 1 for details. Additionally, please, note that Rab7 is a small GTPase involved in the maturation of late endosomal compartments and lysosomes, but not in that of early endosomes.

Major point 5. Colcemid treatment reduced Vero infection by some 40%. This is a rather weak effect to build a scenario implying an early to late endosome trafficking step in infection. And the authors suggest that TMPRSS2 pos cells (Caco2) also use some late endosome processsing steps for infection since these cells are also somewhat sensitive to colcemid, albeit the effect is even weaker than in Vero cells. This claim stands on very thin ice.

Author response:

Colcemid disrupts the microtubule network that is, at least in part, required for the trafficking of late endosomal vesicles. It is well known that it is hard to completely abolish the trafficking of late endosomal vesicles with perturbants such as colcemid or Rab7 mutants (Huotari and Helenius, 2011, EMBO J; Mercer et al., Annu. Rev. Biochem, 2010). In general, perturbation of late endosomal maturation results in a relatively low decrease in infection by late-penetrating viruses (Khor et al., 2003, Traffic; Quirin et al., 2008, Virology; Lozach et al., 2010, Cell Host Microbe). Therefore, the nearby-50% decrease of infection in Vero cells can be already seen as a strong effect. Only the use of a combination of various strategies to impair late endosomal maturation make it possible to draw a conclusion. It is exactly what we propose with our study. Our claim that SARS-CoV-2 relies on late endosomal maturation for the infection of TMPRSS2- cells, i.e., Vero and A549* cells, is based on the following results:

1. SARS-CoV-2 infection requires cathepsin L that is located in late endosomal compartments.
2. Rab7 dominant negative mutant impairs infection by almost 50%.
3. Colcemid impairs infection by almost 50%.
4. MG-132, which depletes the free ubiquitin in the cytosol required for late endosomal maturation, completely blocks infection at extremely low concentrations (IC_{50} < 16 nM)
5. The $t_{1/2}$ of SARS-CoV-2 penetration into TMPRSS2- cells was > 45 min, which is in line with a fusion from late endosomes or lysosomes.
6. SARS-CoV-2 infection depends on endosomal acidification, which is required by endolysosomal proteases for the activation of the spike.

In Caco-2 and Calu-3 cells that both endogenously express TMPRSS2, the inhibition of infection by colcemid was effectively weaker. Hence our hypothesis that colcemid caused a decrease in infection, not directly by blocking the maturation of late endosomes, but, for example, by disrupting the maturation and functions of the upstream early endosomes. Our hypothesis that SARS-CoV-2 infection does not rely on late endosomal trafficking for the infection of TMPRSS2+ cells, i.e., Calu-3 and Caco-2 cells, is further supported by our new results showing that SARS-CoV-2 infection in A549* cells became resistant to MG-132 when TMPRSS2 was expressed (Fig. 5H). This is now further discussed in lines 336-345 as follow:

"*Inhibitors of LE maturation, i.e., colcemid and MG-132, also reduced SARS-CoV-2 infection in a dose-dependent manner in TMPRSS2+ cells (Calu-3 and Caco-2 cells) (Fig. 5E and 5F). Although the inhibition was significant, the decrease in infection caused by colcemid was barely 25-30% (Fig. 5E). The IC50 values of MG-132 were one to three logs higher in TMPRSS2+ cells than in TMPRSS2- cells (Fig. 5F and Table 1). As shown in Fig. 5G, infection was not readily detectable in Calu-3 and Caco-2 cells when MG-132 was added at 2 hpi. Additionally, MG-132 had no effect on SARS-CoV-2 infection of TMPRSS2-overexpressing A549* cells (Fig. 5H). This result contrasted with the infection of parental A549* cells, which decreased with increasing amounts of drug (Fig. 5H). Taken together, these data suggested that the TMPRSS2-dependent SARS-CoV-2 entry pathway does not rely on LE maturation.*"

However, our results do not formally allow to determine whether virus fusion occurs at the plasma membrane or an early endosomal compartment. We cannot exclude that the virus requires

sorting into early endosomal compartments for full activation and fusion. This important point is now discussed under the light of our new results in lines 522-548 as follow:

"*It is clear from our data that, in the presence of TMPRSS2, SARS-CoV-2 did not rely on endosomal acidification and late endosomal maturation for infectious penetration. Concanamycin B, which specifically inhibits vATPases and elevates endosomal pH, affected UUKV, an enveloped virus that penetrates host cells by acid-activated membrane fusion (Lozach et al., 2010), but did not affect SARS-CoV-2 infection. MG-132, known to divert IAV and UUKV away from LEs (Khor et al., 2003, Lozach et al., 2010), was no longer able to impede SARS-CoV-2 infection when TMPRSS2 was overexpressed. This was consistent with reports that TMPRSS2 processes CoV S and other substrates at or near the plasma membrane (Kleine-Weber, Elzayat et al., 2018, Wang & Xiang, 2020), i.e., at neutral pH. Using aprotinin, we found that half of the bound viral particles had completed the TMPRSS2-dependent step within 5-10 min. We cannot completely exclude the possibility that aprotinin was not instantaneously effective when it was added to the infected cells. In this case, the timing of the TMPRSS2-requiring step was therefore faster. SARS-CoV-2 activation and penetration would then likely take place at the plasma membrane following proteolytic activation, as proposed for hCoV-229E and MERS-CoV (Qian, Dominguez et al., 2013, Shirato et al., 2017).*

An alternative scenario is that SARS-CoV-2 is sorted into the endocytic machinery regardless of TMPRSS2 expression. The time course of the TMPRSS2-requiring step resembled that of cargo sorting into EEs, approximately 5-10 min (Huotari & Helenius, 2011). Another observation supporting this hypothesis was that colcemid partially hampered infection. This drug perturbs LE maturation by disrupting the microtubule network and in turn causes EE accumulation and dysfunction (Lozach et al., 2010). Such a strategy has been proposed for reoviruses, which use a similar uptake pathway but different trafficking pathways depending on whether viral particles are activated or not (Boulant, Stanifer et al., 2013). As is the case for other CoVs (Wang & Xiang, 2020), additional functional investigations are required to determine exactly where, whether from the plasma membrane or from EEs, SARS-CoV-2 enters the cytosol of TMRPSS2+ cells, as well as whether the processing of the S protein is followed by transport of the virus to downstream organelles for penetration."

Major point 6. The proteasomal inhibitor MG132 reduced Vero infection. The authors speculate that this is due to depletion of free ubiquitin. Again, quite a daring conclusion in the absence of additional data. More experiments are required to support this claim.

Author response:

Please, see our response to the previous point.

Other point 7: Line 61: I think this statement is not entirely correct as viruses also transmit to other cells by formation of syncytia without using their envelope.

Author response:

Thanks for this comment, the reviewer is correct. However, viral particle-free cell-to-cell transmission of the viral genetic material through synapse or syncytium formation can only occur if hosts, tissues, or cell monolayers are first infected by viral particles. In the context of the introduction, it is matter of enveloped viral particles, and in this respect, to gain access to the cytosol, enveloped viral particles must fuse their envelope with the cell membrane.

Other point 8: Line 70: would is 'act only once'?

Author response:

Priming and activation of class-I viral fusion proteins are irreversible steps, and class-I viral fusion proteins act only once (Harrisson SC, 2015, Virology).

Other point 9: How did they define moi?

Author response:

The multiplicity of infection (MOI) is defined as the average number of infectious viral particles per cell. The MOI was given for SARS-CoV-2 based on the titer determined by plaque-forming unit assays for Vero cells. It is now described in line 648 as follow:

"*The MOI was assigned for SARS-CoV-2 according to the titer determined on Vero cells.*"

Other point 10: Fig2 A,B,C; Fig 4A, D; Fig6A; Fig7C: How can an infection exceed 100%? I am lost.

Author response:

In these experiments, the data are normalized to the samples where the perturbants were omitted, i.e., the mock-treated controls are set to 100% and the other values are provided proportional to this baseline. It happens that values exceed 100% when perturbants cause an enhancement of infection relative to mock-treated controls.

Other point 11: Does the rt-qPCR assay measure subgenomic RNA as well? This matters as different cell lines may produce different subgenomic RNAs.

Author response:

We are sorry, but we do not understand the reviewer's concern as the only qRT-PCR-based results were shown in Figure 1F and intended to quantify the production of newly synthetized virus genomic RNA. These results were part of the experiments aiming to determine the timing of the SARS-CoV-2 life cycle in our four cell lines. They were further confirmed by the detection of newly synthetized viral nucleoprotein by immunofluorescence staining and flow cytometry analysis and correlated with the kinetic of infectious progeny production (Fig. 1C to 1G).

Other point 12: Is aprotinin a peptidic protease inhibitor taken up into cells, and acting in endosomal vesicles?

Author response:

The only information we could find was in the report by Tai and colleagues (2020, Science Advances). These authors suggest that aprotinin is poorly internalized into the endocytic machinery, although no quantification was provided in this study.

1st Revision - Editorial Decision 9th Jun 2021

Dear Pierre-Yves,

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by referees #1 and 2 and their comments are provided below. As you can see the referees appreciate the introduced changes. Referee #2 also indicates that that it would have been better to use a genetic KO to complement the inhibitor experiments. I see that you did add data on A549* cells (overexpress TMPRSS2 in a negative cell) as suggested by referee #1 to go about addressing this point. Can you make sure that you carefully discuss the issue of using inhibitors in the point-by-point response and in the manuscript text.

Taking everything into consideration, I am pleased to let you know that we will move forward with the acceptance of the study for publication here.

Before I can send you the formal acceptance letter you need to take care of the following issues in a final round of revision.

- Please upload high resolution figure files.

- We are missing a data availability section. This is the place to enter accession numbers etc. As far as I can see no data is generated that needs to be deposited in a database. If this is correct please state: This study includes no data deposited in external repositories. Please place it after the Materials and methods and before Acknowledgements

- please double check the reference list - there are some citations with more than 10 authors listed.

- we don't allow data not shown - page 7.

- Please make sure that the funding information is listed both in the manuscript and submission system

- There are figure calls out missing to Fig S3B-D, Fig S5 and S7 panels. Please double check.

- The supplemental files should be either added as expanded view figures (max 5 EV figures) or added to an appendix. Please also see our author guidelines. Please also make sure you correct the figure labeling and call out in text.

- Table 1 should be a word file, or included in the manuscript file.

- We encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. It would be great if you could provide me with a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure? The PDF files should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files.

- We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

- We also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels). You can also use something from the figures if that is easier.

- Our publisher has done their pre-publication check on your manuscript. When you log into the manuscript submission system you will see the file "Data edited manuscript file". Please take a look at the word file and the comments regarding the figure legends and respond to the issues.

That should be all - let me know if you need any further input from me

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
https://bit.ly/EMBOPressFigurePreparationGuideline

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors:
https://www.embopress.org/page/journal/14602075/authorguide

The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 7th Sep 2021.

Link Not Available

Referee #1:

the authors have addressed my concerns and the MS has been significantly improved

Referee #2:

The authors have provided a detailed rebuttal to the points of concerns raised and have satisfied most points. The key criticism that remains, and was made by all three reviewers, is that conclusions are drawn from comparing cell lines that were infected with different numbers of viral particles and treated with different inhibitor concentrations. I agree with the other reviewers that it would be better and cleaner to use genetic KO.

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Heidelberg, 16.06.2021

Dear Dr Dumstrei,

We are pleased with your decision to accept, in principle, our manuscript EMBOJ-2021-107821R. On behalf of all the authors, I thank you and the reviewers for the evaluation of our work.

We have now addressed all the editorial requests in a point-by-point response, which follows this letter. We have also enclosed a separate point-by-point response to Reviewers 1 and 2 at the end of this document. Please, note that we reused the Word file named “Data edited manuscript file” to make the requested changes. Finally, we uploaded the Excel file “Author checklist” again because the reference for the antibody against the SARS-CoV-2 spike was missing.

We hope that the modifications will fulfill your expectations and those of the journal. Do not hesitate to contact us should you need additional information.

We are looking forward to hearing from you.

Yours sincerely,

Pierre-Yves Lozach, PhD

Editorial requests

1 - Please upload high resolution figure files.

Author response:

We have now uploaded figure files with a resolution of 768 dpi.

2 - We are missing a data availability section. This is the place to enter accession numbers etc. As far as I can see no data is generated that needs to be deposited in a database. If this is correct please state: This study includes no data deposited in external repositories. Please place it after the Materials and methods and before Acknowledgements

Author response:

A data availability section has been placed after the Materials and Methods and before the Acknowledgements as follow:

"*This study includes no data deposited in external repositories.*"

3 - please double check the reference list - there are some citations with more than 10 authors listed.

Author response:

The reference style has been updated with the latest EMBO J style available for Endnote.

4 - we don't allow data not shown - page 7.

Author response:

The reference to the data not shown has been removed.

5 - Please make sure that the funding information is listed both in the manuscript and submission system

Author response:

All the funding information is now listed both in the manuscript and in the submission system.

6 - There are figure calls out missing to Fig S3B-D, Fig S5 and S7 panels. Please double check.

Author response:

These figures are now well cited. They have also been renamed to reflect their distribution either in the expanded view figures or in the appendix.

7 - The supplemental files should be either added as expanded view figures (max 5 EV figures) or added to an appendix. Please also see our author guidelines. Please also make sure you correct the figure labeling and call out in text.

Author response:

Figures S3, S6, S7, and S8 now appear as expanded view figures in a single PDF file, i.e., Figures EV1, EV2, EV3, and EV4. Figures S1, S2, S4, S5, S9, and S10 now appear in the Appendix, i.e., Appendix Figures S1, S2, S3, S4, S5, and S6. The citations to these figures have been updated accordingly.

8 - Table 1 should be a word file, or included in the manuscript file.

Author response:

Table 1 is now included in the manuscript file, after the references and before the figure legends.

9 - We encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. It would be great if you could provide me with a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure? The PDF files should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files.

Author response:

We have uploaded the source data as an Excel file, which includes among others the original, uncropped, and unprocessed scans of immunoblots.

10 - We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

Author response:

Please, find here the general summary and the bullet points:

"*Ideally, preventing SARS-CoV-2 spread requires approaches targeting the early steps of infection. This study shows that SARS-CoV-2 infects cells through distinct, mutually exclusive entry routes (e.g., pH independent vs. pH dependent) and highlights the importance of the protease expression patterns of target host cells for sorting SARS-CoV-2 into either pathway.*"

- *The host cell surface protease TMPRSS2 determines the SARS-CoV-2 entry pathway when it is expressed in target cells*
- *In the presence of TMPRSS2, SARS-CoV-2 uses a fast pH-independent route to enter and infect cells*
- *In cells lacking TMPRSS2 expression, SARS-CoV-2 relies on a slow acid-activated late endosomal pathway for infection, and endosomal acidification is required for endolysosomal proteases priming viral fusion*
- *Proteolytic processing is both sufficient and necessary for SARS-CoV-2 fusion*

11 - We also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels). You can also use something from the figures if that is easier.

Author response:

We have now uploaded a graphical abstract (JPEG file, 550 x 400).

12 - Our publisher has done their pre-publication check on your manuscript. When you log into the manuscript submission system you will see the file "Data edited manuscript file". Please take a look at the word file and the comments regarding the figure legends and respond to the issues.

Author response:

We have responded to the issues of the pre-publication check directly in the text following their comments.

Peer Referee #1 comments

the authors have addressed my concerns and the MS has been significantly improved

Author response:

We would like to thank once again Reviewer #1 for the time spent on the evaluation of our work. We are sincerely pleased that the insightful and constructive comments of this reviewer have helped to significantly improve our study.

Peer Referee #2 comments

The authors have provided a detailed rebuttal to the points of concerns raised and have satisfied most points. The key criticism that remains, and was made by all three reviewers, is that conclusions are drawn from comparing cell lines that were infected with different numbers of viral particles and treated with different inhibitor concentrations. I agree with the other reviewers that it would be better and cleaner to use genetic KO.

Author response:

First, we would like to thank Reviewer 2 for their time and comments. With all due respect to Reviewer 2, Reviewers 1 and 3 asked for (i) the MOI used for each experiment, (ii) a clarification about how the MOI was defined and how the concentration of a few inhibitors was chosen, and (iii) cytotoxicity tests for the drugs used in the study. Finally, Reviewers 1 and 3 suggested either a gain-of-function approach or a KO approach. We extensively addressed all these points, including those of Reviewer 2, both in the text and adding new results. Briefly, infecting different cell types with the same number of viral particles while they have diverse sensitivities to SARS-CoV-2 infection would have made it impossible to compare results whether TMPRSS2 is expressed or not. We think that infecting the same number of cells is here the most appropriate strategy. Regarding drug concentrations, we made consistent all the different inhibitor concentrations in the revised manuscript and carefully discussed this point in our responses to reviewers, which overall have fully convinced Reviewers 1 and 3. Hence, we do not consider it necessary to discuss this point further. We agree with Reviewer 2 that a KO approach would have ideally complemented our study, but this was not possible in the time available to revise the manuscript. As recommended by Reviewers 1 and 3, we have instead developed a gain-of-function approach and added a complete set of new data, i.e., Figures 4A to 4D, 5H, 6E, 6F, 7C, 7D, EV3, and EV4.

2nd Revision - Editorial Decision 18th Jun 2021

Dear Pierre-Yves,

Thanks for submitting the revised manuscript to The EMBO Journal. I have now had a chance to take a look at everything and all looks good.

I am therefor very pleased to accept the manuscript for publication here.

Congratulations on a nice study!

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here: https://emboj.embopress.org/about#Transparent_Process

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If you have any questions, please do not hesitate to call or email the Editorial Office. Thank you for your contribution to The EMBO Journal.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Pierre-Yves Lozach
Journal Submitted to: The EMBO Journal
Manuscript Number: 2021-107-821

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical methods were used to determine sample size. We determined the sample size so that the size was sufficient to reach the statistical significance when different conditions were assessed. Sample sizes and statistical tests used in the study are clearly written in the methods and figure legends.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	This study does not involve animals.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data were excluded from the analyses.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No specific randomization approach was used. Though the study only involves virus clones and cell lines, viruses and cells were however pseudo-randomly assigned per condition.
For animal studies, include a statement about randomization even if no randomization was used.	This study does not involve animals.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	This is not relevant to our field for obvious biosafety reasons. The virus studied in this investigation requires a biosafety level 3 laboratory, and investigators needed to know what they were working with. The investigators were therefore not blinded during data collection or analysis.
4.b. For animal studies, include a statement about blinding even if no blinding was done	This study does not involve animals.
5. For every figure, are statistical tests justified as appropriate?	Statistical tests are described in the figure legends where necessary.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The statistical tests were performed based on the assumption that values are normally distributed but it was not assessed. A full description of the statistical tests and parameters (e.g., type of test, one- or two-sided, mean, standard deviation etc.) are present in the figure legend and the Mehods section.
Is there an estimate of variation within each group of data?	Flow cytometry analysis are representative of at least two individual experiments and are shown as the mean of two or more biological replicates acquired for, at least, 5'000 cells. Results from virus titration and qRT-PCR are representative of at least two independent experiments and are given as the mean of triplicates. Replication is clearly written in the methods and figure legends.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

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http://biomodels.net/miriam/
http://jji.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	The assumption was made that the variance is similar between the groups that were statistically compared.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The mouse monoclonal antibody (mAb) against the SARS-CoV-2 nucleoprotein (NP) was purchased from Sino Biologicals (40143-MM05). The rabbit polyclonal antibody (pAb) against the SARS-CoV-2 spike was obtained from Thermo Fischer Scientific (PA1-41165). The rabbit pAb against TMPRSS2 and actin was from Abcam (ab92323) and Sigma (A2066), respectively. The mouse mAbs against cathepsin L and alpha-tubulin were purchased from Thermo Fisher Scientific (BMS1032) and Sigma (T5158), respectively. The goat pAb against EF2 was obtained from Santa Cruz (SC-13004). The rabbit pAb U2 detect all the Uukuniemi virus (UUKV) structural proteins and was first described by Lozach et al., 2011, Cell Host Microbe. The mAb 8B11A3 was used to detect the UUKV nucleoprotein and was a kind gift from the Ludwig Institute for Cancer Research (Sweden) (Lozach et al., 2010, Cell Host Microbe). The mouse mAb against the glycoprotein E2 of Semliki forest virus was kindly provided by Prof. Margaret Kielian (Albert Einstein College, USA) (Kielian et al., 1990, J Virol).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The A549 human epithelial lung cells expressing ACE2 and/or TMPRSS2 were a generous gift from Prof. Ralf Bartenschlager (University Hospital Heidelberg, Germany) (Steuten et al., 2021, ACS Infect Dis). All the other cell lines were originally purchased from ATCC, namely the Vero monkey kidney epithelial cells (ATCC CRL 1586), the Caco-2 human colorectal epithelial cells (ATCC HTB-37), and the Calu-3 human lung epithelial cells (ATCC HTB-55). BHK-21 cells were also originally ordered from ATCC and were a kind gift from Prof. Ari Helenius (ETH Zurich, Switzerland) (Lozach et al., 2010, Cell Host Microbe). All cells were visually authenticated by their morphology, and in addition, BHK-21, Vero, and A549 cells were genetically confirmed.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	This study does not involve animals.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	This study does not involve animals.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	This study does not involve animals.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	This study does not involve human subjects.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	This study does not involve human subjects.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	This study does not involve human subjects.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	This study does not involve human subjects.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	This study does not involve human subjects.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	This study does not involve human subjects.
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	This study does not involve human subjects.

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	No data from this study were deposited in a public database.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	No data from this study were deposited in a public database. All relevant data are included in the paper and the supplementary information files. The material and data that support the findings of this study are also available from the corresponding author upon reasonable request.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	This study does not involve human clinical and genomic datasets.
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	This study does not involve computational models.

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	Our study does not fall under dual use research restrictions.