nature portfolio

Peer Review File

IFITM3 Deficiency Drives SARS-CoV-2 Adaptation While Preserving Variant-Specific Traits

Corresponding Author: Dr Jacob Yount

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The MS from Yount lab has compared the derivation of mouse adapted derivatives of the Beta an Omicron BA.4 in mice knocked out from the antiviral protein IFITM3 that both viruses are sensitive to. After 10-20 passages in ifitm3-/- mice as compared to wt mice, both beta and BA4 selected viruses grew to higher titres in mouse lungs without losing their VoC-defining mutations, implying that mIFITM3 acts as a barrier to the efficient selection of mouse adapted SARS CoV-2. Analysis of adaptive mutations implicated several non-spike mutations as specific to the IFITM3-/- selected viruses. MA-beta has a higher virulence than MA10 (Wuhan mouse adapted) or MA-BA.4. This correlated with lung damage and inflammation and a multitude of cellular and inflammatory markers reminiscent of the pathogenesis of the parental viruses in humans, thus suggesting that IFITM3-/- mice are a better choice for the more rapid selection of experimental systems for studying VoC pathogenesis.

I have had a tough time reviewing this paper. The quality of results, presentation and characterization of the pathogenesis of these mouse-adapted viruses is very high indeed. To the extent that I have very little to criticize here or suggest with respect the extant data. It is also very impressive that the adapted viruses mimic their parental VoCs so well. And also that it moves away from ACE2tg mice that are inherently problematic for pathogenesis studies. However, the paper is observational at present. There is no real understanding from the virus side about what made the difference other than growing to higher titres in mice lacking IFITM3. As such I am a little confused as to what the authors want to conclude from these studies, and what these data tell us about pathogenic mechanisms in COVID19 severity.

• While it is very interesting that passage in an IFITM3 KO mouse selects more readily for mouse adapted SARS-CoV-2, presumably through increased replicative capacity, what this tells us about IFITM3, and SARS-CoV-2 is not so clear. Might this be true of any antiviral protein for which the murine homologue exerts a greater antiviral activity than the human protein? Are the passaged viruses from the wt vs the KO differentially sensitive to mIFITM3 in culture?
• The pathogenesis work is very nice and highly detailed. However, what the paper doesn't address is what were the determinants in the viruses which were necessary or sufficient for mouse adaptation. So, while it is very interesting that the more efficient mouse adaptation with IFITM3-/- mice yields beta and omicron viruses that reflect virulence differences in humans, there is no data for why this is from the viral genetics. This is of course a question that requires molecular clones of the mouse adapted viruses that is beyond the scope here, but it does limit the general interest of the manuscript in my opinion.
• Given that there is evidence from the MCMV system that mIFITM3 is involved in orchestrating DC responses independent of entry restriction, do the mouse-adapted viruses cause similar pathogenesis in IFITM3 -/- mice?

Reviewer #2

(Remarks to the Author)
Denz and colleagues investigated the evolution of the SARS-CoV-2 Beta and Omicron BA.4 variants in wildtype and IFITM3 KO mice. They show that IFITM3 deficiency in mice is associated with increased replication and pathogenesis, as well as accelerated selection of viral genome mutations. They further report that the Beta variant caused significant lung damage, while Omicron BA.4 showed high nasal replication with more limited lung spread and inflammation. The authors also examined the host`s transcriptional response in the lung to infection, and identified alterations in factors regulating cilia

function, extracellular matrix remodeling, and neutrophil recruitment. They conclude that IFITM3 deficiency accelerates viral adaptation but does not alter the distinct characteristics of different SARS-CoV-2 variants.

The manuscript is well-written and the results are clearly presented. However, as outlined below, my main concerns are the novelty and significance of the findings. Previous studies have shown that IFITM3 limits SARS-CoV-2 replication. Additionally, a number of earlier studies have investigated viral adaptations during serial passage of SARS-CoV-2 in mice. The finding that increased replication accelerates viral adaptation is expected and likely not specific for IFITM3. Finally, the species-specificity and the functional consequences of the mutations remain largely speculative.

Specific points

1. While the authors acknowledge that accelerated mutation rates are linked to increased replication, the title and phrasing throughout the manuscript suggest a specificity for IFITM3, which is misleading. This should be changed.

2. The authors convincingly show that the selected mutations increase SARS-CoV-2 replication fitness in mice. However, the conclusion that they are species-specific is not fully warranted, as it remains uncertain whether these alterations also enhance viral replication fitness in other species.

3. While not all relevant previous studies can be cited, some critical references seem to be missing. It has been previously shown that serial passage of SARS-CoV-2 in mice, is associated with adaptive mutations in the SARS-CoV-2 genome, in spike (Gu et al., Science 2020; Ueki et al., Viruses 2024), as well as in the M, N and ORF8 proteins (Rathnasinghe et al., Nat. Comm. 2022). These mutations, observed across variants including Beta, were shown to contribute to lineage-specific phenotypic differences and increased pathogenicity (Willet et al., Commun. Biology 2024; Sun et al., Nat. Comm. 2020). These and other studies provided insights into how SARS-CoV-2 evolves in mice as well as the functional impact of some changes (Leist et al., Cell 2020; Zhang et al., Cell Discovery 2021; Muruato et al., PLOS Biology 2021; Beer et al., JEM 2022). It is difficult to assess whether the adaptations identified in the current study have already been previously observed and analyzed.

4. Similarly, it has been previously reported that host transcriptional responses during SARS-CoV-2 infection leads to cilia dysfunction, extracellular matrix remodeling, and neutrophil recruitment and that these processes contribute to respiratory pathology and exacerbating outcomes (for example, Nunnari et al. Experimental Cell Research 2020; He et al., Protein & Cell 2020; Gutman et al., Viruses 2020; Robinot et al., Nat. Comm. 2021). The most relevant previous studies should be cited and their results discussed and compared to the present data.

5. Analyses of the impact of some identified mutations, such as Nsp9 T35I, Nsp4 A307V and ORF8 F120V, on the function of the respective viral proteins or their contribution to replication and pathogenesis would increase the significance of the study.

Minor points

1. Human and mouse IFITM3 share limited homology, and this limitation should be mentioned.

2. A549 cells overexpressing ACE2 (Figure 1e, i) are useful. However, human cell lines that do not express artificially high levels of ACE2, such as Calu-3, likely represent better models for human airway infection.

3. Different Omicron variants vary greatly in sequence and properties. The authors should specify to which one they refer to in the abstract and elsewhere.

Reviewer #3

(Remarks to the Author)
This manuscript builds on a previous work on IFITM3-dependent viral adaptation—originally demonstrated with influenza virus—by applying the approach to SARS-CoV-2 variants. It shows that IFITM3 deficiency lowers the barrier to interspecies adaptation, while not entirely novel (due to the authors own work in IAV) it is extended here and cements the concept. Specifically, the study applies the adaptation concept to both Beta and Omicron variants of SARS-CoV-2, exploring how these viruses evolve with distinct impacts on viral tropism, inflammatory responses, and variant-specific pathogenic properties. The creation of novel, mouse-adapted Omicron strains is a highly useful resource for the field. Additionally, the thorough transcriptomic analysis provides supportive mechanistic insights into the nuances of variant-specific inflammation and immune recruitment Again, this is a valuable community resource and the insights made do shed light on pathogenic outcomes with variants.

I actually have very few technical comments. This is because the manuscript uses very well established methodology and approaches, though I do not want to give the impression that the work here is in any way routine. And in regards the transcriptomic analysis I may be less qualified to comment and hope other reviewers are. I believe the manuscript is ultimately worthy of publication.

However, I recommend that the authors more clearly acknowledge and delineate how this study extends—and differs from—their previous work with influenza virus (Denz et al., Nat. Commun., 2024). Positioning this work more in the context of a

broader host-adaptation framework, with a particular focus pandemic preparedness, would strengthen things. A major point of revision would be whether the authors think that IFITM3 is unique in this ability to act as a zoonotic barrier, or would we see the same for any other ISG?

Further, the recent mBio study by the same group analyzing GWAS data and highlighting a potential role for IFITM proteins in regulating the severity of emerging viral infections adds further weight to the specific significance of IFITM3 in viral emergence. Nonetheless, this broader relevance raises an important question: are these findings unique to IFITM proteins, or could similar host adaptation outcomes be observed with the loss of other key interferon-stimulated genes (ISGs)?

At the most stringent level, the specific significane of IFITM over any other ISG would be addressed experimentally, though this is of course an unrealistic request. Addressing this point thoroughly in the discussion would help define IFITM3's unique role. These comments are separate to the many insights the authors have generated in regards pathogenesis by manipulating this axis to generate adapted variants, and again I am supportive.

There are some minor points outlined below:

Figure 1 and Methods Clarity: The inter-individual variability is unclear in some of the viral titer data. Were results presented as pooled lung homogenates or individual measurements? It may have been preferable to pool samples to reduce variability, depending on the aim. Please clarify how many mice were used in each experiment and whether a single mouse line was used throughout. These details are currently underexplained in both the text and the methods.
Line 143: The reference to the extended data figure should be to AddFig2d, not AddFig2c.
Figure 3D (Viral dissemination to the heart): The authors interpret detection of virus in the heart as evidence of broader dissemination, but could this simply reflect higher overall viral load? This could be clarified by examining viral presence in additional control organs to rule out non-specific spread due to high titres. Or at least commenting with alternative interpretation.
Line 240: The statement that higher viral titres in lungs suggest a greater output per infected cell is of interest. However, this interpretation would be stronger with supporting data, such as single-cell–level qPCR or quantification normalised to cell number. This could be mentioned as a future direction if not feasible here.
Line 245 (Whole-body plethysmography): There is no explanation of how plethysmography was performed. A brief description in the methods would be useful, as this technique requires specific interpretation.
Line 315: The Extended Data Table referenced appears to be missing. Please ensure it is included and appropriately cited in the final version.
Line 330 and 332: The text currently refers to Fig. 6b, but should refer to Fig. 6c.
Line 333: This reference should be to Fig. 6b rather than Fig. 6c.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addreesed my concerns within the scope of the current study

Reviewer #2

(Remarks to the Author)
Yount and colleagues have addressed some of my concerns by significantly expanding the discussion. Amongst others, they now provide an example illustrating that loss of antiviral factors may also result in viral attenuation rather than adaptation; add a comprehensive table of known SARS-CoV-2 mouse-adaptive mutations and relevant references; clarify that these mutations may also enhance replication in species other than mice. In addition, they acknowledge the importance of analyzing individual mutations, such as Nsp9 T35I, Nsp4 A307V, and ORF8 F120V but note that this is beyond the scope of the present study. The authors also added new experimental data including IFITM3-KO infection phenotypes and additional validation in human cells. Finally, the authors now provide more detailed comparison with existing mouse-adaptation studies and highlight newly identified mutations.

The authors made an effort to address the reviewer`s issues and the manuscript is improved. My main concern remains the novelty and significance of the findings. As now acknowledged in the manuscript, a number of earlier studies have shown that IFITM3 limits SARS-CoV-2 replication and investigated viral adaptations during serial passage of SARS-CoV-2 in mice. The example of how the effects of OAS1 on influenza virus infection contrast the present findings is interesting. However, as noted by the authors without experimental comparisons to other antiviral pathways, the claim that IFITM3 plays a distinct or privileged role remains suggestive rather than definitive. Finally, the functional significance of specific adaptive mutations remains to be determined and hence conclusions about how these mutations shape viral fitness remain speculative. Altogether, the systematic analysis of adaptive changes in SARS-CoV-2 during passaging in IFITM3-deficient mice is of some interest and the viral variants useful for further study. Nonetheless, the advance beyond previous work seems limited.

Minor points

I previously suggested to clarify that “Human and mouse IFITM3 share limited homology”. In their reply, the authors state that the homology is substantial (67% identity). I agree. However, especially since the authors now emphasize sequence variations and deficiency of IFITM3 in humans it seems useful to specify how human IFITM3 compares to mouse IFITM3. They should also consider to clarify that true “knockout-like” IFITM3 disruption is vanishingly rare while hypomorphic alleles with modest functional effects are surprisingly common.

It was difficult to see where exactly changes have been made since the line numbers in the rebuttal seem incorrect.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

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I have had a tough time reviewing this paper. The quality of results, presentation and characterization of the pathogenesis of these mouse-adapted viruses is very high indeed. To the extent that I have very little to criticize here or suggest with respect the extant data. It is also very impressive that the adapted viruses mimic their parental VoCs so well. And also that it moves away from ACE2tg mice that are inherently problematic for pathogenesis studies. However, the paper is observational at present. There is no real understanding from the virus side about what made the difference other than growing to higher titres in mice lacking IFITM3. As such I am a little confused as to what the authors want to conclude from these studies, and what these data tell us about pathogenic mechanisms in COVID19 severity.

• While it is very interesting that passage in an IFITM3 KO mouse selects more readily for mouse adapted SARS-CoV-2, presumably through increased replicative capacity, what this tells us about IFITM3, and SARS-CoV-2 is not so clear. Might this be true of any antiviral protein for which the murine homologue exerts a greater antiviral activity than the human protein? Are the passaged viruses from the wt vs the KO differentially sensitive to mIFITM3 in culture?

We agree that the enhanced adaptation of SARS-CoV-2 in IFITM3 KO mice likely reflects greater replicative capacity and increased opportunity for the virus to explore adaptive sequence space.

In response to the reviewer's specific points:

1) Our laboratory's evidence indicates that murine and human IFITM3 both restrict SARS-CoV-2 infections (Shi, *EMBO J*, 2020). It is possible, as the reviewer notes, that loss of other ISGs with anti-SARS-CoV-2 activity could similarly facilitate interspecies adaptation, and we have added a paragraph to the Discussion considering this possibility (Lines 561-582). We emphasize, however that IFITM3 is of particular interest because naturally occurring human IFITM3 deficiencies are relatively common (20% of Chinese individuals and 4% of Europeans). Thus, our finding that IFITM3 deficiency accelerates coronavirus adaptation has direct and relevant implications for human SARS-CoV-2 susceptibility and public health. As we also discuss, loss of other restriction factors may remove selective pressures that could instead attenuate viral fitness in WT hosts, making generalized statements difficult without empirical testing as we have done here for IFITM3.

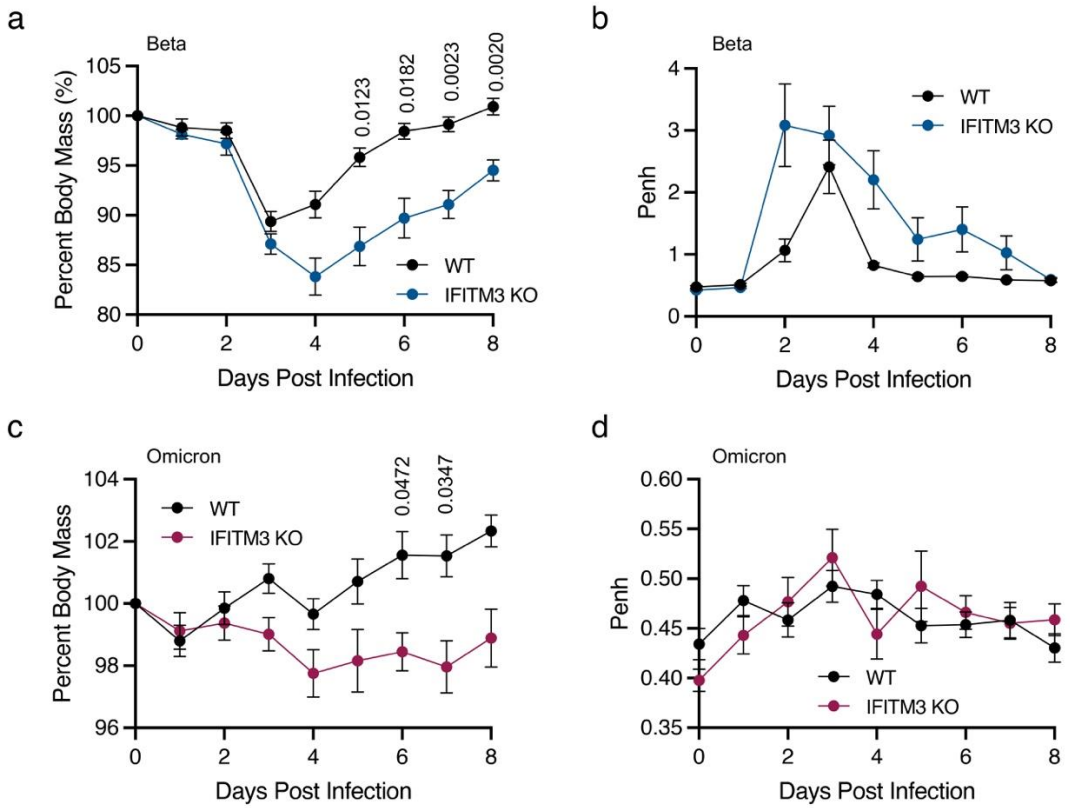
2) To assess whether viruses passaged in IFITM3 KO mice exhibit altered IFITM3 sensitivity, we measured infections by parental and KO passage 20 viruses in cells expressing murine or human IFITM3. We observed no differences in susceptibility of the passaged viruses to IFITM3 restriction (Extended Data Fig. 2). These results suggest that enhanced adaptation in IFITM3-deficient mice stems from increased replication and mutation exploration rather than selection for IFITM3-specific evasion (which indeed would not be expected to arise in the absence of IFITM3-mediated selective pressure).

• The pathogenesis work is very nice and highly detailed. However, what the paper doesn't address is what were the determinants in the viruses which were necessary or sufficient for mouse adaptation. So, while it is very interesting that the more efficient mouse adaptation with IFITM3-/- mice yields beta and omicron viruses that reflect virulence differences in humans, there is no data for why this is from the viral genetics. This is of course a question that requires molecular clones of the mouse adapted viruses that is beyond the scope here, but it does limit the general interest of the manuscript in my opinion.

We agree with the reviewer that identifying the functional significance of the individual mutations arising in our mouse-adapted strains would be highly interesting from a virological perspective. However, as the reviewer notes, generating each individual viral clone and performing the mechanistic characterization required to dissect their specific effects is beyond the current scope of this study. To begin addressing this important question, we have assembled a comprehensive resource for the field, a table summarizing all SARS-CoV-2 mouse-adaptive mutations reported to date (Extended Data Table 1). This analysis highlights the S protein as a frequent hotspot for mouse adaptation and reveals that numerous mutational pathways can enhance SARS-CoV-2 replication in mice. Notably, many of the mutations identified in our study appear to be novel, though our data, together with at least two independent reports, reproducibly identify N R32 and M T7 as recurring sites for mouse adaptative substitutions, suggesting strong selective pressures at these amino acid positions.

• Given that there is evidence from the MCMV system that mIFITM3 is involved in orchestrating DC responses independent of entry restriction, do the mouse-adapted viruses cause similar pathogenesis in IFITM3 -/- mice?

We performed a pilot infection experiment in WT and KO mice with our mouse-adapted Beta and Omicron strains to explore the reviewer's question. Although mice available in our colony were ~13 wks of age and showed less pathogenesis than we see in 6-8 wk old mice, we nonetheless observed more weight loss and worse lung function in IFITM3 KOs infected with the Beta strain. Likewise, KOs infected with Omicron lost a small amount of weight while WTs gained weight over the course of infection. No changes in lung function were observed for Omicron infections, consistent with the weak effects of Omicron on lungs. Overall, these results provided expected results, consistent with our *in vitro* experiments, indicating IFITM3 is restrictive of the adapted viruses and we did not investigate this concept further with additional cohorts of mice. We provide the pilot results here for the reviewer.



Reviewer Figure 1: IFITM3 KO mice show worsened infection phenotypes with mouse-adapted SARS-CoV-2. A-B 13 week old WT and IFITM3 KO mice were infected with 10^5 TCID50 KO P20 Beta SARS-CoV-2. **A** Weight loss (Error bars represent SEM, comparisons were made using the Mann-Whitney test). **B** Daily whole-body plethysmography enhanced pause (Penh) measurements (dots represent averages of individual mice, non-significant by Mann-Whitney test). **C-D** WT and IFITM3 KO mice were infected with 10^5 TCID50 KO P20 Omicron SARS-CoV-2. **A** Weight loss (Error bars represent SEM, comparisons were made using the Mann-Whitney test). **B** Daily whole-body plethysmography enhanced pause (Penh) measurements (dots represent averages of individual mice, non-significant by Mann-Whitney test). N = 10 for all groups in **A-D**.

Reviewer #2 (Remarks to the Author):

Denz and colleagues investigated the evolution of the SARS-CoV-2 Beta and Omicron BA.4 variants in wildtype and IFITM3 KO mice. They show that IFITM3 deficiency in mice is associated with increased replication and pathogenesis, as well as accelerated selection of viral genome mutations. They further report that the Beta variant caused significant lung damage, while Omicron BA.4 showed high nasal replication with more limited lung spread and inflammation. The authors also examined the host`s transcriptional response in the lung to infection, and identified alterations in factors regulating cilia function, extracellular matrix remodeling, and neutrophil recruitment. They conclude that IFITM3 deficiency accelerates viral adaptation but does not alter the distinct characteristics of different SARS-CoV-2 variants.

The manuscript is well-written and the results are clearly presented. However, as outlined below, my main

concerns are the novelty and significance of the findings. Previous studies have shown that IFITM3 limits SARS-CoV-2 replication. Additionally, a number of earlier studies have investigated viral adaptations during serial passage of SARS-CoV-2 in mice. The finding that increased replication accelerates viral adaptation is expected and likely not specific for IFITM3. Finally, the species-specificity and the functional consequences of the mutations remain largely speculative.

Specific points

1. While the authors acknowledge that accelerated mutation rates are linked to increased replication, the title and phrasing throughout the manuscript suggest a specificity for IFITM3, which is misleading. This should be changed.

Our experiments utilized IFITM3 KO versus WT mice and thus our results are specific for IFITM3. As discussed above, these are important and relevant experiments given the prevalence of naturally occurring human IFITM3 deficiencies. Moreover, our past work showed that passaging of influenza virus through STAT1 KOs, which also allowed enhanced viral replication, resulted in viral attenuation rather than adaptation (Denz et al., *Nat Commun*, 2024). We now include a new paragraph in the Discussion addressing the concept that loss of some antiviral restriction mechanisms could result in viral attenuation rather than adaptation due to absence of selective pressures. Overall, we contend that the effects of each specific immunodeficiency on viral adaptation must be empirically determined as done in our study.

2. The authors convincingly show that the selected mutations increase SARS-CoV-2 replication fitness in mice. However, the conclusion that they are species-specific is not fully warranted, as it remains uncertain whether these alterations also enhance viral replication fitness in other species.

Our reference to species specificity in this context refers to the observation that mouse-adaptive mutations do not enhance replicative fitness in human cells as demonstrated by our experiments in human A549 cells and new Supplemental Figures 1 and 2 showing no replicative advantage for mouse adapted viruses in primary human bronchial epithelial cell cultures or human HEK293T-ACE2 cells. We do not find that our language excludes the possibility that the mouse-adaptive mutations may enhance replication in other species, particularly other rodents, and we now discuss this possibility (Lines 532-536).

3. While not all relevant previous studies can be cited, some critical references seem to be missing. It has been previously shown that serial passage of SARS-CoV-2 in mice, is associated with adaptive mutations in the SARS-CoV-2 genome, in spike (Gu et al., Science 2020; Ueki et al., Viruses 2024), as well as in the M, N and ORF8 proteins (Rathnasinghe et al., Nat. Comm. 2022). These mutations, observed across variants including Beta, were shown to contribute to lineage-specific phenotypic differences and increased pathogenicity (Willet et al., Commun. Biology 2024; Sun et al., Nat. Comm. 2020). These and other studies provided insights into how SARS-CoV-2 evolves in mice as well as the functional impact of some changes (Leist et al., Cell 2020; Zhang et al., Cell Discovery 2021; Muruato et al., PLOS Biology 2021; Beer et al., JEM 2022). It is difficult to assess whether the adaptations identified in the current study have already been previously observed and analyzed.

We thank the reviewer for this comment as it inspired us to assemble a resource table of mutations seen in all published SARS-CoV-2 mouse adaptation studies (Extended Data Table 1). This reveals that 1) there are multiple evolutionary paths to mouse adaptation, 2) the adaptive mutations seen in our study are largely novel, and 3) that there are at least two reproducibly observed mouse-adaptive hotspots that may represent particularly interesting targets for future study (N R32 and M T7). We now cite the listed references and all other mouse adaptation studies identified in our comprehensive literature searches.

4. Similarly, it has been previously reported that host transcriptional responses during SARS-CoV-2 infection leads to cilia dysfunction, extracellular matrix remodeling, and neutrophil recruitment and that these processes contribute to respiratory pathology and exacerbating outcomes (for example, Nunnari et al. Experimental Cell Research 2020; He et al., Protein & Cell 2020; Gutman et al., Viruses 2020; Robinot et al., Nat. Comm. 2021). The most relevant previous studies should be cited and their results discussed and compared to the present data.

We have now discussed these references in the context of our current study (Lines 512-522).

5. Analyses of the impact of some identified mutations, such as Nsp9 T35I, Nsp4 A307V and ORF8 F120V, on the function of the respective viral proteins or their contribution to replication and pathogenesis would increase the significance of the study.

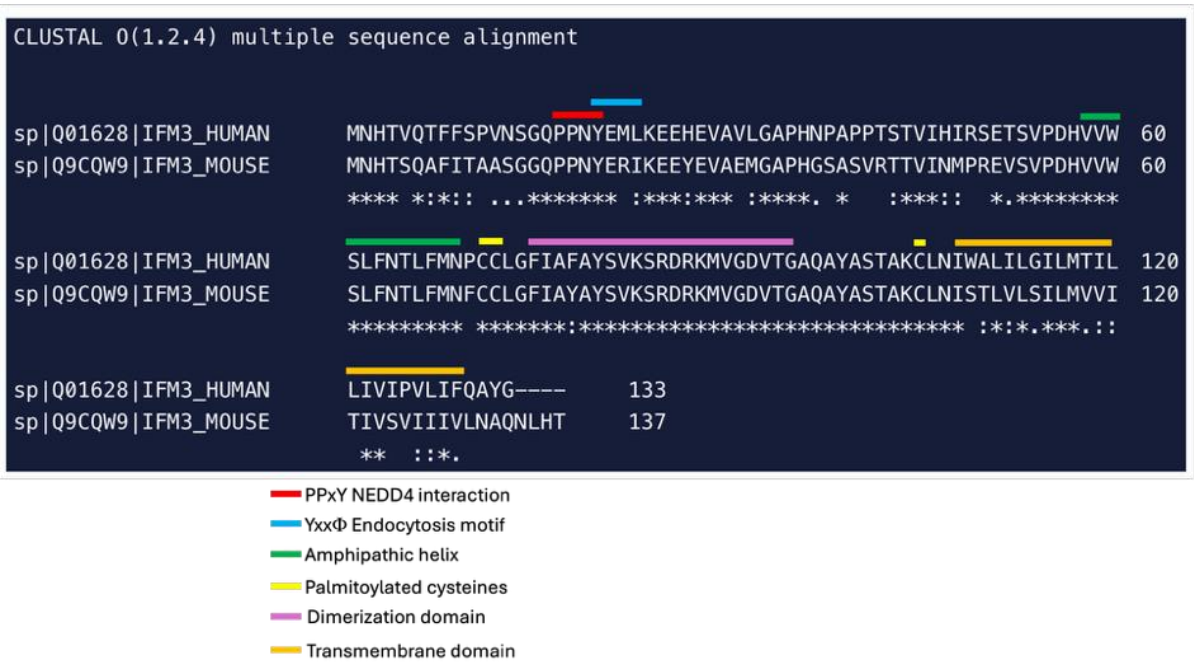
We agree that mechanistic analyses of individual adaptive mutations such as Nsp9 T35I, Nsp4 A307V, and ORF8 F120V would provide additional insight into their specific contributions to viral replication and pathogenesis. However, these studies would require extensive virological, pathogenesis, and mechanistic characterization that is beyond the scope and resources of the present work. Our goal here was to identify and compare the efficiency of adaptation under defined experimental conditions, and to develop new virus strains to be used as tools to study pathogenesis in mice. We have added a statement to the Discussion to emphasize that these mutations represent exciting targets for future functional studies (Lines 547-551). Moreover, our results add to the growing understanding of mouse-adaptive mutational hotspots as revealed in our comprehensive compiling of known mouse-adaptive SARS-CoV-2 mutations (Extended Data Table 1).

Minor points

1. Human and mouse IFITM3 share limited homology, and this limitation should be mentioned.

We appreciate the comment, but human and mouse IFITM3 in fact share substantial homology. The proteins are 67% identical overall, with most divergence confined to the N-terminal disordered segment and the C-terminal transmembrane region. Despite the N- and C-terminal divergence, key functional motifs in these regions are conserved: the YxxΦ endocytic localization motif, PPxY motif (NEDD4 ubiquitin ligase interaction motif), and a hydrophobic C-terminal transmembrane helix. Moreover, the central functional core responsible for antiviral activity, including IFITM3's amphipathic helix, conserved palmitoylated cysteines, and residues implicated in dimerization, is highly conserved between species. We

provide below an alignment for the reviewer highlighting the critical conserved regions of mouse and human IFITM3.



2. A549 cells overexpressing ACE2 (Figure 1e, i) are useful. However, human cell lines that do not express artificially high levels of ACE2, such as Calu-3, likely represent better models for human airway infection.

We have now added confirmatory data using primary human bronchial epithelial cells differentiated at air-liquid interface (Extended Data Fig 1) and human HEK293T-ACE2 cells (Extended Data Fig 2). These data further support the conclusion that our highly mouse-adapted viruses do not gain replicative fitness in human cells.

3. Different Omicron variants vary greatly in sequence and properties. The authors should specify to which one they refer to in the abstract and elsewhere.

We refer to Omicron BA.4 in the abstract and provide the complete name of the BA.4 Omicron strain in the Materials and Methods section with the catalogue number from BEI Resources (hCoV-19/USA/MD-HP30386/2022; NR-56803).

Reviewer #3 (Remarks to the Author):

This manuscript builds on a previous work on IFITM3-dependent viral adaptation—originally demonstrated with influenza virus—by applying the approach to SARS-CoV-2 variants. It shows that IFITM3 deficiency lowers the barrier to interspecies adaptation, while not entirely novel (due to the authors own work in IAV) it is extended here and cements the concept. Specifically, the study applies the

adaptation concept to both Beta and Omicron variants of SARS-CoV-2, exploring how these viruses evolve with distinct impacts on viral tropism, inflammatory responses, and variant-specific pathogenic properties. The creation of novel, mouse-adapted Omicron strains is a highly useful resource for the field. Additionally, the thorough transcriptomic analysis provides supportive mechanistic insights into the nuances of variant-specific inflammation and immune recruitment Again, this is a valuable community resource and the insights made do shed light on pathogenic outcomes with variants.

We thank the reviewer for the kind recognition of the importance of our work. We address remaining comments together below since there are interrelated.

I actually have very few technical comments. This is because the manuscript uses very well established methodology and approaches, though I do not want to give the impression that the work here is in any way routine. And in regards the transcriptomic analysis I may be less qualified to comment and hope other reviewers are. I believe the manuscript is ultimately worthy of publication.

However, I recommend that the authors more clearly acknowledge and delineate how this study extends—and differs from—their previous work with influenza virus (Denz et al., Nat. Commun., 2024). Positioning this work more in the context of a broader host-adaptation framework, with a particular focus pandemic preparedness, would strengthen things. A major point of revision would be whether the authors think that IFITM3 is unique in this ability to act as a zoonotic barrier, or would we see the same for any other ISG?

Further, the recent mBio study by the same group analyzing GWAS data and highlighting a potential role for IFITM proteins in regulating the severity of emerging viral infections adds further weight to the specific significance of IFITM3 in viral emergence. Nonetheless, this broader relevance raises an important question: are these findings unique to IFITM proteins, or could similar host adaptation outcomes be observed with the loss of other key interferon-stimulated genes (ISGs)?

At the most stringent level, the specific significane of IFITM over any other ISG would be addressed experimentally, though this is of course an unrealistic request. Addressing this point thoroughly in the discussion would help define IFITM3's unique role. These comments are separate to the many insights the authors have generated in regards pathogenesis by manipulating this axis to generate adapted variants, and again I am supportive.

We agree that the loss of any ISG capable of potently restricting SARS-CoV-2, as seen with IFITM3, could theoretically facilitate accelerated adaptation by allowing greater exploration of randomly arising mutations. We have now acknowledged this possibility, albeit with some nuance, in the Discussion. However, as emphasized in our response to reviewer #1, reviewer #2, and now elaborated further in the manuscript, we believe IFITM3 holds particular importance. First, IFITM3 is one of the few ISGs for which relatively common human deficiencies exist. As the reviewer notes, the published literature and our recent *mBio* review article describe that ~20% of Chinese individuals and ~4% of Europeans carry IFITM3 polymorphisms associated with decreased antiviral activity, underscoring the significance of specifically investigating how IFITM3 deficiency alters infection and evolutionary dynamics.

A second ISG with frequent human variation is, 2’-5’-oligoadenylate synthetase 1 (OAS1), which harbors a splice-site polymorphism (rs10774671) known to reduce antiviral activity against SARS-CoV-2 (Wickenhagen, *Science*, 2021). However, the consequences of OAS1 loss on viral adaptation would likely

differ from those of IFITM3 loss. IFITM3 restricts viral entry, so its absence primarily allows infection of cells that would otherwise be resistant, thereby expanding the replicating population and thus the opportunity for host adaptation. OAS1 acts later by sensing viral dsRNA replication intermediates and activating RNase L to degrade viral RNA. In OAS1-deficient hosts, replication would increase, potentially promoting diversification and adaptation, but there may simultaneously be less selective pressure to minimize dsRNA production or evade OAS1 signaling. Consequently, viruses adapted in OAS1-deficient hosts might acquire traits that are maladapted or attenuated in WT hosts, where intact OAS1/RNase L activity would again constrain replication. We observed a comparable outcome in a prior study when passaging influenza virus through STAT1-deficient mice, where the loss of interferon signaling unexpectedly led to viral attenuation rather than enhanced adaptation. These viruses developed polymerase variants associated with greater interferon induction in WT hosts (Denz et al., *Nat Commun*, 2024). These findings support the notion that while the absence of any strong restriction factor can expand the viral mutational landscape, the direction and consequence of that evolution depend on the specific antiviral pathway removed. We therefore contend that the unique role of IFITM3 as a zoonotic barrier, and the broader effects of other ISGs on viral evolution, can only be resolved through empirical testing.

We thank the reviewer for prompting this valuable discussion and have incorporated a new paragraph into the discussion outlining these considerations using OAS1 as an example.

There are some minor points outlined below:

Figure 1 and Methods Clarity: The inter-individual variability is unclear in some of the viral titer data. Were results presented as pooled lung homogenates or individual measurements? It may have been preferable to pool samples to reduce variability, depending on the aim. Please clarify how many mice were used in each experiment and whether a single mouse line was used throughout. These details are currently underexplained in both the text and the methods.

Figure 1 caption and methods under “virus stocks and in vitro infection” were updated to clarify that each dot is representative of an individual mouse or independent cell culture infection (Lines 613-615). For every experiment, the number of mice used is present in the figure caption. Samples were not pooled in any experiments in this study.

Line 143: The reference to the extended data figure should be to AddFig2d, not AddFig2c.

This has been corrected.

Figure 3D (Viral dissemination to the heart): The authors interpret detection of virus in the heart as evidence of broader dissemination, but could this simply reflect higher overall viral load? This could be clarified by examining viral presence in additional control organs to rule out non-specific spread due to high titres. Or at least commenting with alternative interpretation.

We added new data for two other organs with relevance to SARS-CoV-2 infections, namely brain and pancreas tissue from MA10, Beta, and Omicron infected mice. We show that replication competent virus

was not present in these tissues (Fig 3e-f). This may suggest that cardiac dissemination represents organ-selective viral spread rather than general viremia.

Line 240: The statement that higher viral titres in lungs suggest a greater output per infected cell is of interest. However, this interpretation would be stronger with supporting data, such as single-cell–level qPCR or quantification normalised to cell number. This could be mentioned as a future direction if not feasible here.

We tempered our statement here (Line 296) and now cite three references showing particularly robust replication of Omicron in comparison to other variants. It is particularly interesting to note from these references that Omicron replicates to heightened levels in epithelial cells of the large airways, but is attenuated for replication in deeper lung parenchymal tissue, consistent with our results.

Line 245 (Whole-body plethysmography): There is no explanation of how plethysmography was performed. A brief description in the methods would be useful, as this technique requires specific interpretation.

The methods were updated to reflect how whole-body plethysmography was performed and analyzed (Lines 720-726).

Line 315: The Extended Data Table referenced appears to be missing. Please ensure it is included and appropriately cited in the final version.

This table (now Extended Data Table 2) is submitted and appropriately referenced.

Line 330 and 332: The text currently refers to Fig. 6b, but should refer to Fig. 6c.

This has been corrected.

Line 333: This reference should be to Fig. 6b rather than Fig. 6c.

This has been corrected.

REVIEWER RESPONSE

Reviewer #1 (Remarks to the Author):

The authors have addreesed my concerns within the scope of the current study

Reviewer #2 (Remarks to the Author):

Yount and colleagues have addressed some of my concerns by significantly expanding the discussion. Amongst others, they now provide an example illustrating that loss of antiviral factors may also result in viral attenuation rather than adaptation; add a comprehensive table of known SARS-CoV-2 mouse-adaptive mutations and relevant references; clarify that these mutations may also enhance replication in species other than mice. In addition, they acknowledge the importance of analyzing individual mutations, such as Nsp9 T35I, Nsp4 A307V, and ORF8 F120V but note that this is beyond the scope of the present study. The authors also added new experimental data including IFITM3-KO infection phenotypes and additional validation in human cells. Finally, the authors now provide more detailed comparison with existing mouse-adaptation studies and highlight newly identified mutations.

The authors made an effort to address the reviewer`s issues and the manuscript is improved. My main concern remains the novelty and significance of the findings. As now acknowledged in the manuscript, a number of earlier studies have shown that IFITM3 limits SARS-CoV-2 replication and investigated viral adaptations during serial passage of SARS-CoV-2 in mice. The example of how the effects of OAS1 on influenza virus infection contrast the present findings is interesting. However, as noted by the authors without experimental comparisons to other antiviral pathways, the claim that IFITM3 plays a distinct or privileged role remains suggestive rather than definitive. Finally, the functional significance of specific adaptive mutations remains to be determined and hence conclusions about how these mutations shape viral fitness remain speculative. Altogether, the systematic analysis of adaptive changes in SARS-CoV-2 during passaging in IFITM3-deficient mice is of some interest and the viral variants useful for further study. Nonetheless, the advance beyond previous work seems limited.

Minor points

I previously suggested to clarify that “Human and mouse IFITM3 share limited homology”. In their reply, the authors state that the homology is substantial (67% identity). I agree. However, especially since the authors now emphasize sequence variations and deficiency of IFITM3 in humans it seems useful to specify how human IFITM3 compares to mouse IFITM3. They should also consider to clarify that true “knockout-like” IFITM3 disruption is vanishingly rare while hypomorphic alleles with modest functional effects are surprisingly common.

It was difficult to see where exactly changes have been made since the line numbers in the rebuttal seem incorrect.

We appreciate the reviewer's clarification and have added the following statement in the Discussion:

With respect to the relevance of our findings to human genetics, human and mouse IFITM3 share approximately 67% amino acid identity, indicating substantial conservation. This conservation is particularly strong in motifs associated with endocytic trafficking[1, 2], protein turnover[3, 4], and antiviral activity[5, 6, 7, 8]. Nevertheless, species-specific differences could influence IFITM3 expression and antiviral function. Complete, KO-like IFITM3 disruption in humans has not been reported, whereas the hypomorphic alleles with partial but significant loss of function or expression are relatively common[9]. In this context, IFITM3 KO mice provide a well-defined and unambiguous system to delineate the impact of IFITM3 deficiency on SARS-CoV-2 adaptation. Although human hypomorphic alleles produce partial loss of function phenotypes, their effects are predicted to be directionally consistent with those observed in IFITM3 KO mice.

1. Jia R, *et al.* Identification of an endocytic signal essential for the antiviral action of IFITM3. *Cell Microbiol* **16**, 1080-1093 (2014).

2. Chesarino NM, McMichael TM, Hach JC, Yount JS. Phosphorylation of the antiviral protein interferon-inducible transmembrane protein 3 (IFITM3) dually regulates its endocytosis and ubiquitination. *J Biol Chem* **289**, 11986-11992 (2014).

3. Yount JS, Karssemeijer RA, Hang HC. S-palmitoylation and ubiquitination differentially regulate interferon-induced transmembrane protein 3 (IFITM3)-mediated resistance to influenza virus. *J Biol Chem* **287**, 19631-19641 (2012).

4. Chesarino NM, McMichael TM, Yount JS. E3 Ubiquitin Ligase NEDD4 Promotes Influenza Virus Infection by Decreasing Levels of the Antiviral Protein IFITM3. *PLoS Pathog* **11**, e1005095 (2015).

5. Yount JS, *et al.* Palmitoylome profiling reveals S-palmitoylation-dependent antiviral activity of IFITM3. *Nat Chem Biol* **6**, 610-614 (2010).

6. Chesarino NM, *et al.* IFITM3 requires an amphipathic helix for antiviral activity. *EMBO Rep* **18**, 1740-1751 (2017).

7. Rahman K, Datta SAK, Beaven AH, Jolley AA, Sodt AJ, Compton AA. Cholesterol Binds the Amphipathic Helix of IFITM3 and Regulates Antiviral Activity. *J Mol Biol* **434**, 167759 (2022).

8. Rahman K, Wilt I, Jolley AA, Chowdhury B, Datta SAK, Compton AA. SNARE mimicry by the CD225 domain of IFITM3 enables regulation of homotypic late endosome fusion. *Embo j* **44**, 534-562 (2025).

9. Denz PJ, Yount JS. IFITM3 variants point to a critical role in emergent virus infections. *mBio* **16**, e0334724 (2025).