nature portfolio

Peer Review File

A single mutation may contribute to accelerated evolution of SARS-CoV-2 toward Omicron

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
1. The authors are overstating the role of epistasis, and in some examples, misusing the term. Epistasis refers to interactions between genetic loci where one mutation affects the impact of another. However, the authors seem to use it as a catch-all term for any mutation that facilitates additional changes, without strong evidence that T492I actually directly modifies the effects of other mutations in a classic epistatic manner. While T492I may be a precursor mutation facilitating subsequent evolutionary change, the claims of strong epistasis lack direct genetic interaction data. A more cautious interpretation would be that T492I increases mutation rates and replication efficiency, indirectly setting the stage for Omicron-like mutations to arise, rather than directly determining their effects. Could the authors please revisit the use of the term and arguments throughout the manuscript.

2. The manuscript is focused on T492I as the key ‘driver mutation’ but does not sufficiently compare its influence to other notable mutations, such as N501Y or ΔSGF, that are also known to shape SARS-CoV-2 evolution. While some experiments include N501Y and ΔSGF (e.g., Figure 4g-i), their relative impacts are not deeply analyzed beyond infectivity metrics. N501Y has been shown to increase ACE2 binding affinity and interact epistatically with other spike mutations to enhance infectivity. Does T492I have a stronger or weaker effect in this regard? Furthermore, would viruses with both T492I and N501Y show different fitness outcomes than what would be expected from adding their individual effects? That would be evidence of true epistasis.

3. Please provide more details regarding the sequencing methodology, primarily amplicon and library construction conditions (limiting cycle numbers are critical for accurate variant calling) as well as, read length to help assess the validity of haplotype reconstructions.

4. Could the authors elaborate further on the difference evolutionary trajectories delta and omicron have taken, despite carrying this key same change in NSP4? The authors have focused so much on the role in driving Omicron emergence (convergently) without actually accounting for the distinct evolutionary origins of the two variants, and the relatively limited mutational landscape for delta.

5. Furthermore, the authors briefly suggest that T492I might have emerged from early SARS-CoV-2 variants and facilitated the rapid adaptation of Omicron-like lineages. This provides scope to incorporate additional phylogenetic analysis comparing the prevalence of T492I in historical strains preceding Omicron to consider the proposed hypothesis in greater detail (for example, the prevalence and detailed timings of T492I emergence). This would strengthen arguments for/against convergent evolution.

6. Could the individual variant phylogenies for each re-sequencing experiment be combined in some form to explore the relative trajectories each virus took, as well as, the relative diversity accumulated?

7. Perhaps, this question is a little naïve, but what do the authors feel is the major force shaping the evolution of the viruses in these in vitro experiments? The authors take much about immune selection/evasion, mutation rates, and I wondered how much this work actually translated from cell culture to transmission and epidemiological scales, and herd immunity which is the primary driver seems at these population scales. Some reflection perhaps in the manuscript?

8. The “strong predisposition driven by T492I, like a wormhole-tunnel effect in Astrocosmology”. This is a bit sensational. Can we find a more apt analogy?

Reviewer #2

(Remarks to the Author)
Sequence analysis and evolutionary evidence have indicated that SARS-CoV-2 mutation T492I in the nonstructural protein 4 (NSP4) may enhance viral replication and alter nonstructural protein cleavage. Following up on this observation Lin et al design and carry out a number of ingenious evolve-and-resequence experiments to investigate T429I potential to drive evolution through both direct and epistatic mechanisms. Their result shows a statistically significant excess of heteregozigosity and genetic diversity in viral strains following the emergence of the T429I mutants compared to the wild-type.

The demonstration of a direct mechanism linking a mutation in NSP4 to hypermutations in the downstream genome, including the Spike protein, is of central importance both to understand SARS-CoV-2's evolution of pathogenicity and for molecular evolutionary theory in general.

I have no major remarks. Experiments are solid and results robust. As a minor remark, I would suggest the authors, in agreement with the latest guidelines from international static associations, to provide odds ratios rather (or together with) the p-values of their statistical comparisons.

Reviewer #3

(Remarks to the Author)
Herein Lin and colleagues report that a single polymorphism NSP4 mutation T492I drives a rapid phenotypic adaption and a predisposition to rapid emergence of SARS-CoV-2 Omicron-like variants. Overall the authors present an interesting case for the T492I as a fitness gain that enabled accelerated evolution during the pandemic. Whilst this is an intriguing hypotheses, the pandemic to date has been marked by almost yearly cases of newly emerging variants that have significant accumulation in changes compared to those in circulation. This started with the first VOCs and then continued with the 2022 Omicron lineages (BA2/BA5), 2023 lineages (XBB) and now 2024 lineages (JN.1). Where and how each batch of new lineages appear is unknown and could be a case of poor genomic surveillance in many countries and/or the intrahost evolution and subsequent spread and release into the community. So my initial comment is that SARS-CoV-2 lineages have appeared over time, with what "appears" to be accelerated evolution from time to time, with lineages appearing not only with significant accumulation of changes in their genomes, but also with significant transmission fitness advantages. It also must be noted that Omicron is simply a WHO name that began with the arrival of BA.1 & the appearance of many dominant lineages with significant shifts in entry and immune evasion have appeared since that time. So overall, whilst the authors may have identified an interesting fitness advantage in isolated with NSP4 mutation T492I, I would conclude in my opinion it is one of many changes to the virus that have now locked in for it to sustain a fitness advantage in the face of strong population immunity. Specific comments are as follows:

-Lines 62-67. Replication of Omicron in primary cells is complex, the virus still uses TMPRSS2 in primary cultures but in a manner that has yet to be recapitulated in cell lines. The commentary here largely refers to initial work using engineered cell lines that simply only express ACE2 and TMPRSS2. In this setting, the default entry pathway for Omicron is endocytosis, as the conditions limit S1/S2 cleavage. The same does not proceed in primary cells that represent the respiratory tract. The latter is complex and goes beyond the entry factors of ACE2 and TMPRSS2 alone.
- Whilst T492I may give the virus a fitness advantage and be retained in many contemporary circulating variants, it must be noted that this change has appeared in early variants of the pandemic. Eg. A Clade A in early 2020: GISAID = hCoV-19/USA/GA-EHC-108D/2020. Thus the appearance this early and the subsequent delay in the appearance of Omicron lineages is a disconnect in the commentary within by the authors. Whilst the accelerate replication kinetics is well known in Omicron lineages, it really represents a sum of parts and a change in a viral entry mechanism that has been poorly resolved in vitro. For instance how and why the Omicron lineages outcompete earlier pre-Omicron lineages in the nasal cavity cannot be attributed to a shift in viral entry purely based on endocytosis of the virus, as all SARS CoV-2 viruses to date can enter via this pathway. In addition a singular change to NSP4 may give viruses additional fitness advantages, but so to do changes in many other viral ORFs and especially in the Spike glycoprotein.
-Methodologically the use of Calu3s as a means to mimic rounds of transmission is at odds with how the virus has evolved. In fact, Omicron lineages actually grow poorly in lung derived cells such as Calu3s and primary type 2 alveolar cells. In contrast Omicrons have grown to rapidly and efficiently target the ciliated nasal epithelia and this alone points to regions of Spike rather than NSP4.
Line-473. The emergence of the first Omicron may not have been rapid, if there was intrahost evolution for a significant periods of time and then emergence in the community. This is often seen in cryptic waste water lineages that appear from individuals but are not widely circulating in the community. The fact this has happened on almost a yearly basis from BA.2 to BA.2.75/XBB to BA.2.86/JN1, supports mechanisms beyond NSP4. Sure, NSP4 maybe one of many changes that have led to these phenotypes, but overall there are many accumulated changes in the virus that have contributed towards this.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Overall the authors have addressed previous concerns well, especially with the additional experiments.

There are a couple of minor things worth tidying up.

1. Line 47: I just realised "evolvement" would be better replaced with "evolution"
2. Lines 475-479: Some toning down a little here. The historical enrichment of Omicron-related mutations in 492I-bearing mutations is important and consistent with the authors 'predisposition hypothesis' however it is not causation and many T492I-bearing viruses went extinct before Omicron emerged. The authors should perhaps here mention that timing and context matter, and there were likely other additional host and ecological factors that contributed to the emergence and evolution of Omicron.
3. Lines 353-355: Again, maybe take some care to avoid overstating things here. While the data is consistent with positive epistatis, indirect effects such as increased replication rate or cell tropism changes may be contributing to the phenotypic effects... maybe some clarification saying "under the genetic backgrounds tested" or "within the limits of these in vitro models".

Reviewer #2

(Remarks to the Author)
The manuscript describes a series on in vitro evolution experiments to show how a crucial mutation in the SARS-CoV-2 NSP4 gene, T492I, is potentially responsible for accelerating the evolution of the viral genomes leading to the development of more aggressive variants, such as the omicron variants. The revised manuscript is well written and convincingly addresses the critiques of the reviewers by making sure to use the term epistasis in an appropriate way and showing, through sequence analyses of wild type and Delta strains with or without the T492I substitution, that mutated strains accumulate quantitatively more mutations in other genes, thus generating variants similar to the circulating omicron strains. I have no further remarks for the co-authors. This is a robust manuscript that demonstrates the existence and importance of mutation-driven predisposition in viral evolution for SARS-CoV-2 and potentially opens the way to investigate such an important phenomenon in other viruses.

Reviewer #3

(Remarks to the Author)
Thank you to the authors for addressing the concerns I have raised through their reviewed submission. The experiments are extensive and the data is of interest. The changes does drive a fitness gain BUT the virus will always will be the sum of many parts and will evolve to gain fitness under many varying conditions. The appearance of Omicron-like lineages in waste-waster that pre-dates their appearance, supports replication and evolution of Omicron lineages to a very specific tissue environment (intestine). Yes this change may accelerate the virus to get to that point and importantly represents a fitness gain alongside many other changes in the virus that have enabled to persist in the face of high level mature immune responses. The results herein are indeed of interest but are at times oversold to be the driving force of major evolutionary steps in the virus.The mechanism of accelerated evolution through the interaction with cytidine deaminases is also interesting but not well developed. Cytidine deaminases are observed to significantly restrict viral replication through hypermutation (well at least for HIV). So how does the mechanism change here? and if cytidine deaminases were removed from cells, would this render the polymorphism redundant? I'm not requesting the authors do additional experiments, but rather qualify that far more mechanistic work would need to be done to make this claim.

Responses to Reviewer 1

1. The authors are overstating the role of epistasis, and in some examples, misusing the term. Epistasis refers to interactions between genetic loci where one mutation affects the impact of another. However, the authors seem to use it as a catch-all term for any mutation that facilitates additional changes, without strong evidence that T492I actually directly modifies the effects of other mutations in a classic epistatic manner. While T492I may be a precursor mutation facilitating subsequent evolutionary change, the claims of strong epistasis lack direct genetic interaction data. A more cautious interpretation would be that T492I increases mutation rates and replication efficiency, indirectly setting the stage for Omicron-like mutations to arise, rather than directly determining their effects. Could the authors please revisit the use of the term and arguments throughout the manuscript.

Response: We greatly appreciate your suggestions. We revisited the use of the term epistasis throughout the manuscript and have utilized a more cautious interpretation of the impact of NSP4 T492I to other mutations in the revision (in lines 43-46, 82-85, 324-355). The corresponding revision in the Abstract and Introduction parts are shown below.

"Aside from elevated mutation rates and impacts on deaminases, positive epistasis between T492I and adaptive mutations should mechanistically further facilitate the shifts in mutation spectra and indirectly determines the Omicron-predisposing evolution."

"Our previous *in silico* analysis of the highly transmissible Delta sub-variant 21J (with 492I) and the less transmissible variants 21A+21I (with T492) predicted a possibly additive effect of T492I to adaptive mutations in fitness."

Moreover, we followed your suggestions concerning the evidence of epistasis and included additional experiments to evaluate the interaction between T492I and other adaptive mutations. The results (Fig. 4h-s and Supplementary Fig. 5d-i) support positive epistasis between NSP4 T492I and S N501Y, between T492I and Omicron spike mutations (a combination of five spike RBD mutations, 5SM), between N501Y and 5SM, and between T491I and N501Y + 5SM. Based on the additionally included data (Fig. 4 and Supplementary Fig. 5), we have carefully revised the corresponding parts as shown in lines 324-355, 493-505, Fig. 4h-s and Supplementary Fig. 5.

2. The manuscript is focused on T492I as the key 'driver mutation' but does not sufficiently compare its influence to other notable mutations, such as N501Y or ΔSGF, that are also known to shape SARS-CoV-2 evolution. While some experiments include N501Y and ΔSGF (e.g., Figure 4g-i), their relative impacts are not deeply analyzed beyond infectivity metrics. N501Y has been shown to increase ACE2 binding affinity and interact epistatically with other spike mutations to enhance infectivity. Does T492I have a stronger or weaker effect in this regard? Furthermore, would viruses with both T492I and N501Y show different fitness outcomes than what would be

expected from adding their individual effects? That would be evidence of true epistasis.

Response: We thanks a lot for this insightful comment. In accordance with your suggestion, we have included additional experiments to compare the influence of NSP4-T492I and other notable mutations, such as S N501Y or NSP6 ΔSGF. We found that the Delta strains with both T492I and N501Y mutations show a significant additional additive effect on replication and infectivity than the sum of their individual effects (Supplementary Fig. 5a-i). Further, we have included experiments to compare the epistasis effect of T492I and N501Y on the interaction with other spike mutations. Five spike mutations (5SM), namely S373P, K417N, S477N, Q498R, and Y505H were selected according to the sequencing data. As the T492I mutation is located in a nonstructural protein and cannot bind to ACE2 directly, in this context, replication, infectivity and immune evasion capacity were examined. The results show that the viruses with both T492I and 5SM exhibit significantly stronger replication, infectivity (Fig. 4h-m and Supplementary Fig. 5j-l) and immune evasion capacity (Fig. 4n-s and Supplementary Fig. 5m-o) outcomes than that would be expected from the sum of their individual effects, suggesting a positive epistasis between T492I and 5SM. We also identified positive epistasis between N501Y and 5SM and between T492I and N501Y+5SM (Fig. 4h-s). T492I+5SM occasionally exhibit stronger replication and immune evasion capacity than N501Y+5SM (Supplementary Fig. 5j-o). Taken together, these results indicate that NSP4 T492I is capable of inducing positive epistastic effects to adaptive Omicron mutations, similar to the spike mutation N501Y. The corresponding revision is in lines 324-355, 493-505, 816-827, Fig. 4h-s and Supplementary Fig. 5.

3. Please provide more details regarding the sequencing methodology, primarily amplicon and library construction conditions (limiting cycle numbers are critical for accurate variant calling) as well as, read length to help assess the validity of haplotype reconstructions.

Response: Following this suggestion, we included more details regarding the sequencing methodology and relevant experiments in the revised manuscript (lines 588-595), as show below.

“Pair-end genomic sequencing was performed via an Illumina NextSeq 2000 apparatus. NEB Ultra II RNA was used for non-directional (non-strand-specific) library preparation. RNA library construction was performed strictly according to the Product Manual (www.neb.com/en-us/-/media/nebus/files/manuals/manuale7770_e7775.pdf). The temperature/time/cycles for initial denaturation, denaturation, annealing/extension, final extension and hold are 98°C/30 sec/1, 98°C/10 sec/7-15, 65°C/75 sec/7-15, 65°C/5 sec/1 and 4°C/∞, respectively. Based on total RNA input amount, the number of PCR cycles were limited to avoid over amplification. The read length is 111 bp.”

4. Could the authors elaborate further on the difference evolutionary trajectories delta and omicron have taken, despite carrying this key same change in NSP4? The authors have focused

so much on the role in driving Omicron emergence (convergently) without actually accounting for the distinct evolutionary origins of the two variants, and the relatively limited mutational landscape for delta.

Response: We greatly appreciate your suggestions. In the revised manuscript, we elaborated further on the evolutionary difference between Delta and Omicron, such as the distinct evolutionary origins of these two VOCs and the relatively limited mutations for Delta (lines 93-99).

"The VOCs Delta and Omicron both bear the T492I mutation. However, previous research suggests that these two VOCs are attributed to distinct phylogroups and do not share a common ancestry [28,29]. Delta has a relatively limited mutational landscape, compared to Omicron. The new mutations in the Delta variant emerged through multiple stages, a process of acceleration with more and more mutations [30]. T492I may contribute differently to the evolution of these two different VOCs."

5. Furthermore, the authors briefly suggest that T492I might have emerged from early SARS-CoV-2 variants and facilitated the rapid adaptation of Omicron-like lineages. This provides scope to incorporate additional phylogenetic analysis comparing the prevalence of T492I in historical strains preceding Omicron to consider the proposed hypothesis in greater detail (for example, the prevalence and detailed timings of T492I emergence). This would strengthen arguments for/against convergent evolution.

Response: Thank you very much for your suggestions. Following these, we performed phylogenetic analyses of historical strains preceding the spread of Omicron variants to compare the prevalence of Omicron mutations in the strains with and without T492I. The results exhibit that the Omicron spike mutations more frequently emerged in the variants with 492I than that with T492 (Supplementary Fig. 4). We revised the corresponding sentences in lines 256-268, 475-479, 662-675.

6. Could the individual variant phylogenies for each re-sequencing experiment be combined in some form to explore the relative trajectories each virus took, as well as, the relative diversity accumulated?

Response: We appreciate your suggestions. For this issue, we constructed combined phylogenies of different viruses and calculated the diversity accumulated. We found that the populations with 492I accumulated more mutations (2.7~6.9 folds), higher diversities (1.4 ~ 25 folds) and higher percentage of spike mutations than those with T492 (Supplementary Fig3a-d, f). Meanwhile, the phylogenetic relationship between the 45-day and 90-day dominant strains of the populations

with T492I suggest a potentially convergent evolution under the effect of T492I (Supplementary Fig. 3a-d). The corresponding revision is in lines 249-255 and the Supplementary Fig. 3.

7. Perhaps, this question is a little naïve, but what do the authors feel is the major force shaping the evolution of the viruses in these in vitro experiments? The authors take much about immune selection/evasion, mutation rates, and I wondered how much this work actually translated from cell culture to transmission and epidemiological scales, and herd immunity which is the primary driver seems at these population scales. Some reflection perhaps in the manuscript?

Response: Thank you for your comments. The purpose of the in-vitro experiment is to evaluate the effect of the replication-enhancing mutation NSP4 T492 to the evolution of SARS-CoV-2. The results exhibit the capability of T492I to regulate the evolutionary trajectories of SARS-CoV-2 and induce a predisposition toward Omicron mutations. Following your previous suggestion, we included phylogenetic and population genomic analyses on historical data, which agree with the contribution of the T492I-driven predisposition to the emergence of Omicron mutations in an epidemiological level. However, we considered that the major force that shapes the viral evolution is the host/environmental factors (e.g. herd immunity), since the host/environmental factors induce the selection on the mutation-driven phenotypes. The driver mutation and the host/environmental factors should jointly and interactively regulate the evolution of the SARS-CoV-2. We appended these in the revised manuscript in lines 528-534.

8. The “strong predisposition driven by T492I, like a wormhole-tunnel effect in Astrocosmology”. This is a bit sensational. Can we find a more apt analogy?

Response: We appreciate your comments. We have revised the corresponding sentences in lines 506-508.

Responses to Reviewer 2

Sequence analysis and evolutionary evidence have indicated that SARS-CoV-2 mutation T492I in the nonstructural protein 4 (NSP4) may enhance viral replication and alter nonstructural protein cleavage. Following up on this observation Lin et al design and carry out a number of ingenious evolve-and-resequence experiments to investigate T429I potential to drive evolution through both direct and epistatic mechanisms. Their result shows a statistically significant excess of heteregozigosity and genetic diversity in viral strains following the emergence of the T429I mutants compared to the wild-type.

Response: Thank you very much for your positive comments.

The demonstration of a direct mechanism linking a mutation in NSP4 to hypermutations in the downstream genome, including the Spike protein, is of central importance both to understand SARS-CoV-2's evolution of pathogenicity and for molecular evolutionary theory in general.

Response: We greatly appreciate your positive comments.

I have no major remarks. Experiments are solid and results robust. As a minor remark, I would suggest the authors, in agreement with the latest guidelines from international static associations, to provide odds ratios rather (or together with) the p-values of their statistical comparisons.

Response: Thank you very much for your positive comments and insightful suggestion. For this issue, we calculated and included the odds ratios in the revised manuscript, as shown in Figs. 3i, j, 4b, 6c-e and supplementary Figs. 2g-i, 3f.

Responses to Reviewer 3

Herein Lin and colleagues report that a single polymorphism NSP4 mutation T492I drives a rapid phenotypic adaption and a predisposition to rapid emergence of SARS-CoV-2 Omicron-like variants. Overall the authors present an interesting case for the T492I as a fitness gain that enabled accelerated evolution during the pandemic. Whilst this is an intriguing hypotheses, the pandemic to date has been marked by almost yearly cases of newly emerging variants that have significant accumulation in changes compared to those in circulation. This started with the first VOCs and then continued with the 2022 Omicron lineages (BA2/BA5), 2023 lineages (XBB) and now 2024 lineages (JN.1). Where and how each batch of new lineages appear is unknown and could be a case of poor genomic surveillance in many countries and/or the intrahost evolution and subsequent spread and release into the community. So my initial comment is that SARS-CoV-2 lineages have appeared over time, with what "appears" to be accelerated evolution from time to time, with lineages appearing not only with significant accumulation of changes in their genomes, but also with significant transmission fitness advantages. It also must be noted that Omicron is simply a WHO name that began with the arrival of BA.1 & the appearance of many dominant lineages with significant shifts in entry and immune evasion have appeared since that time. So overall, whilst the authors may have identified an interesting fitness advantage in isolated with NSP4 mutation T492I, I would conclude in my opinion it is one of many changes to the virus that have now locked in for it to sustain a fitness advantage in the face of strong population immunity.

Response: Thank you very much for your comments and insightful suggestions. Our study suggests a T492I-driven predisposition to the emergence of Omicron mutations. However, we agree that the major force that shapes the viral evolution is the host/environmental factors (e.g. herd immunity), since the host/environmental factors induce the selection on the mutation-driven phenotypes. The included population genomic analyses on historical data show that Omicron-like variants repeatedly emerged in the strains with T492I and went to loss in the two years preceding the spread of Omicron variants. The loss of Omicron-like variants is possibly due to a low frequency in the population, which results in an uncompetitive relative fitness (the growth rate of a genotype relative to another genotype picked as the standard of comparison) and the purging by genetic drift. It is also possible that that the adaptiveness of Omicron-like variants is comparably lower in 2020 than that in the late of 2021 and the time thereafter, owing to the increased immunized proportion of the human population. Due to the possibility of missing historical samples, we agree that further work is still needed to investigate the evolutionary origin and relevant detailed evolutionary mechanism of the Omicron variants. We included these in lines 513-525, 541-543 of the revised manuscript.

Specific comments are as follows:

-Lines 62-67. Replication of Omicron in primary cells is complex, the virus still uses TMPRSS2 in primary cultures but in a manner that has yet to be recapitulated in cell lines. The commentary here largely refers to initial work using engineered cell lines that simply only express ACE2 and TMPRSS2. In this setting, the default entry pathway for Omicron is endocytosis, as the conditions limit S1/S2 cleavage. The same does not proceed in primary cells that represent the respiratory tract. The latter is complex and goes beyond the entry factors of ACE2 and TMPRSS2 alone.

Response: We greatly appreciate your suggestion. According to this suggestion, we have deleted the commentary referring to initial work. The revised sentences are in lines 60-64.

- Whilst T492I may give the virus a fitness advantage and be retained in many contemporary circulating variants, it must be noted that this change has appeared in early variants of the pandemic. Eg. A Clade A in early 2020: GISAID = hCoV-19/USA/GA-EHC-108D/2020. Thus the appearance this early and the subsequent delay in the appearance of Omicron lineages is a disconnect in the commentary within by the authors. Whilst the accelerate replication kinetics is well known in Omicron lineages, it really represents a sum of parts and a change in a viral entry mechanism that has been poorly resolved in vitro. For instance how and why the Omicron lineages outcompete earlier pre-Omicron lineages in the nasal cavity cannot be attributed to a shift in viral entry purely based on endocytosis of the virus, as all SARS CoV-2 viruses to date can enter via this pathway. In addition a singular change to NSP4 may give viruses additional fitness advantages, but so to do changes in many other viral ORFs and especially in the Spike glycoprotein.

Response: Thank you very much for your suggestions. We included additional phylogenetic and epidemiologic analyses of historical strains (Supplementary Fig. 4). The results show that there used to be Omicron-like strains as early as 2020, and those Omicron-like strains are mostly evolved in the strains with 492I. These Omicron-like strains emerged in 492I strains and went to loss later for multiple times before the emergence and spread of Omicron variants at the beginning of 2022. The loss of these early Omicron-like strains is possibly due to a low frequency in the population, which results in an uncompetitive relative fitness (the growth rate of a genotype relative to another genotype picked as the standard of comparison) and the purging by genetic drift. It is also possible that the adaptiveness of Omicron-like variants is comparably lower in 2020 than that in the late of 2021 and the time thereafter, owing to the herd effect. After the spread of T492I from the mid of 2021, Omicron mutations were introduced more intensively (Supplementary Fig. 4g), which may, together with the increases of the immunised proportion, result in a competitive relative fitness for Omicron-like variants and the emergence of Omicron variants. Aside from the predisposition effect of T492I, the host/environmental factors (such as herd immunity) should be a major force that shapes the viral evolution, since the

host/environmental factors cause the selection pressure and selects the mutation-driven phenotypes. For example, the increased global vaccination coverage may have enhanced the adaptiveness of Omicron-like or Omicron variants. The driver mutation and host/environmental factors should jointly and interactively regulate the evolution of the SARS-CoV-2. The revised sentences are in lines 513-534.

Further, we agree that the Omicron lineages have accelerated replication kinetics, and how and why the Omicron lineages outcompete earlier pre-Omicron lineages in the nasal cavity cannot be attributed to a shift in viral entry purely based on endocytosis of the virus. According to this suggestion, we have deleted the relevant sentences as shown in lines 60-64 of the revised manuscript.

As last, we agree that other viral ORFs and especially the spike glycoprotein give viruses additional fitness advantages, the corresponding revision is in lines 512-514.

-Methodologically the use of Calu3s as a means to mimic rounds of transmission is at odds with how the virus has evolved. In fact, Omicron lineages actually grow poorly in lung derived cells such as Calu3s and primary type 2 alveolar cells. In contrast Omicrons have grown to rapidly and efficiently target the ciliated nasal epithelia and this alone points to regions of Spike rather than NSP4.

Response: We greatly appreciate the reviewer for this insightful comment. Firstly, we fully agree with the reviewer that upper airway is the major site of replication for variant of concerns (VOCs), such as Omicron, and Omicron strains have been reported to rapidly and efficiently target the nasal ciliated epithelial cells (CECs) [1]. It is noteworthy that nasal and upper airway CECs are also the major targets of the early strains of SARS-CoV-2 [2,3]. Moreover, many respiratory viruses, including SARS-CoV-1 [4], influenza [5], parainfluenza (PIV) [6], rhinovirus (RV) [7], and respiratory syncytial virus (RSV) [8], first infect upper airway CECs before spreading the infection to other organs and tissues. Therefore, the emergence of the Omicron strains is most likely not entirely dependent on the Omicron's preference for CECs and ACE2-Spike interactions, although Omicron strains have an extremely high affinity for CECs that highly express ACE2 and proliferate rapidly in such cells. Secondly, we agree that the Omicron lineages show decreased growth kinetics in lung-derived cells, such as Calu-3 and primary type 2 alveolar cells. However, our data show that the strains with Omicron mutations still exhibit a good growth curve on Calu3 cells. This implies that Omicron-like virus could still replicate and amplify normally in lung derived cells. This is consistent with the report of Sakurai et al [9]. We inferred that the processes of replication, assembly, and post-entry budding of the virus may also be important and more closely related to the NSPs, despite the close association of the spike protein with viral invasion and its importance in the viral life cycle. Moreover, the evolved viruses in the in vitro

evolutionary model are not identical to the Omicron strains that are prevalent in the real world. Specifically, there are many Omicron-representative mutations that are not present in the evolved viruses, while the evolved viruses also possess mutations that are not present in the Omicron strains. The evolve-and-resequence experiment in this study merely mimicked an evolutionary process of the original SARS-CoV-2 strain and the data discussed the driving factors in the evolution of original SARS-CoV-2 strain to the Omicron-like strains. The evidence provided in this study supports an evolutionary driving effect of the NSP4 T492I, but does not exclude a role of the spike protein and other proteins in this process. Based on the above, we have modified the manuscript appropriately and discussed this point in the Discussion section of the revised manuscript (lines 463-467, 506-513).

References:

[1] Wu CT, Lidsky PV, Xiao Y, Cheng R, Lee IT, Nakayama T, Jiang S, He W, Demeter J, Knight MG, Turn RE, Rojas-Hernandez LS, Ye C, Chiem K, Shon J, Martinez-Sobrido L, Bertozzi CR, Nolan GP, Nayak JV, Milla C, Andino R, Jackson PK. SARS-CoV-2 replication in airway epithelia requires motile cilia and microvillar reprogramming. Cell. 2023 Jan 5;186(1):112-130.e20.

[2] Hou YJ, Okuda K, Edwards CE, Martinez DR, Asakura T, Dinnon KH 3rd, Kato T, Lee RE, Yount BL, Mascenik TM, Chen G, Olivier KN, Ghio A, Tse LV, Leist SR, Gralinski LE, Schäfer A, Dang H, Gilmore R, Nakano S, Sun L, Fulcher ML, Livraghi-Butrico A, Nicely NI, Cameron M, Cameron C, Kelvin DJ, de Silva A, Margolis DM, Markmann A, Bartelt L, Zumwalt R, Martinez FJ, Salvatore SP, Borczuk A, Tata PR, Sontake V, Kimple A, Jaspers I, O'Neal WK, Randell SH, Boucher RC, Baric RS. SARS-CoV-2 Reverse Genetics Reveals a Variable Infection Gradient in the Respiratory Tract. Cell. 2020 Jul 23;182(2):429-446.e14.

[3] Ahn JH, Kim J, Hong SP, Choi SY, Yang MJ, Ju YS, Kim YT, Kim HM, Rahman MDT, Chung MK, Hong SD, Bae H, Lee CS, Koh GY. Nasal ciliated cells are primary targets for SARS-CoV-2 replication in the early stage of COVID-19. J Clin Invest. 2021 Jul 1;131(13):e148517.

[4] Jia HP, Look DC, Shi L, Hickey M, Pewe L, Netland J, Farzan M, Wohlford-Lenane C, Perlman S, McCray PB Jr. ACE2 receptor expression and severe acute respiratory syndrome coronavirus infection depend on differentiation of human airway epithelia. J Virol. 2005 Dec;79(23):14614-21.

[5] Thompson CI, Barclay WS, Zambon MC, Pickles RJ. Infection of human airway epithelium by human and avian strains of influenza a virus. J Virol. 2006 Aug;80(16):8060-8.

[6] Zhang L, Bukreyev A, Thompson CI, Watson B, Peeples ME, Collins PL, Pickles RJ. Infection of ciliated cells by human parainfluenza virus type 3 in an in vitro model of human airway epithelium. J Virol. 2005 Jan;79(2):1113-24.

[7] Griggs TF, Bochkov YA, Basnet S, Pasic TR, Brockman-Schneider RA, Palmenberg AC, Gern JE. Rhinovirus C targets ciliated airway epithelial cells. Respir Res. 2017 May 4;18(1):84.

[8] Zhang L, Peeples ME, Boucher RC, Collins PL, Pickles RJ. Respiratory syncytial virus infection of human airway epithelial cells is polarized, specific to ciliated cells, and without obvious cytopathology. J Virol. 2002 Jun;76(11):5654-66.

[9] Sakurai Y, Okada S, Ozeki T, Yoshikawa R, Kinoshita T, Yasuda J. SARS-CoV-2 Omicron subvariants progressively

adapt to human cells with altered host cell entry. mSphere. 2024 Sep 25;9(9):e0033824.

Line-473. The emergence of the first Omicron may not have been rapid, if there was intrahost evolution for a significant periods of time and then emergence in the community. This is often seen in cryptic waste water lineages that appear from individuals but are not widely circulating in the community. The fact this has happened on almost a yearly basis from BA.2 to BA.2.75/XBB to BA.2.86/JN1, supports mechanisms beyond NSP4. Sure, NSP4 maybe one of many changes that have led to these phenotypes, but overall there are many accumulated changes in the virus that have contributed towards this.

Response: Thank you very much for your suggestions. We agree that the emergence of the first Omicron may not have been rapid. For this issue, we revised the sentences relevant to the emergence of the first Omicron and included the possibility of the evolvement of Omicron variants in the cryptic waste water lineages. The corresponding revised sentences are in lines 534-541, as shown below:

"Despite the rapid epidemic expansion of Omicron after the spotting this VOC, the evolvement of the first Omicron may not have been rapid. As described above, multiple emerged Omicron-like variants went to loss in the two years after the pandemic of SARS-CoV-2. Moreover, there may be intrahost evolution for a long time before the emergence of Omicron in the community. For example, the cryptic waste water lineages [83,84] may contribute importantly to the emergences of Omicron mutations."

Responses to Reviewer 1

Overall the authors have addressed previous concerns well, especially with the additional experiments.

Response: Thank you very much for your comments and insightful suggestions for the improvement of our manuscript.

There are a couple of minor things worth tidying up.

1. Line 47: I just realized "evolvement" would be better replaced with "evolution"

Response: We appreciate your comments. We have revised the corresponding sentences in line 47.

2. Lines 475-479: Some toning down a little here. The historical enrichment of Omicron-related mutations in 492I-bearing mutations is important and consistent with the authors 'predisposition hypothesis' however it is not causation and many T492I-bearing viruses went extinct before Omicron emerged. The authors should perhaps here mention that timing and context matter, and there were likely other additional host and ecological factors that contributed to the emergence and evolution of Omicron.

Response: Thank you very much for your suggestions. Following this suggestion, we have revised the sentences in lines 475-481 in the revised version, as shown below.

“Population genomic and phylogenetic analyses on historical data support the T492I-induced predisposition to the emergence of Omicron mutations. These suggest that the predisposing mutation NSP4 T492I may contribute importantly to the emergence and spread of Omicron variants. Historically, many T492I-bearing viruses went extinct before the spread of Omicron. Other host/environmental factors may also contribute importantly to the emergence and evolution of Omicron.”

3. Lines 353-355: Again, maybe take some care to avoid overstating things here. While the data is consistent with positive epistasis, indirect effects such as increased replication rate or cell tropism changes may be contributing to the phenotypic effects... maybe some clarification saying "under the genetic backgrounds tested" or "within the limits of these in vitro models".

Response: We appreciate your suggestion. Following this suggestion, we have revised the sentences in lines 353-355 to avoid overstating the implication.

“Taken together, these date suggest that, aside from S N501Y, NSP4 T492I is also capable of inducing a positive epistastic effect to adaptive Omicron mutations within the limits of these in vitro models”

Responses to Reviewer 2

The manuscript describes a series on in vitro evolution experiments to show how a crucial mutation in the SARS-CoV-2 NSP4 gene, T492I, is potentially responsible for accelerating the evolution of the viral genomes leading to the development of more aggressive variants, such as the omicron variants. The revised manuscript is well written and convincingly addresses the critiques of the reviewers by making sure to use the term epistasis in an appropriate way and showing, through sequence analyses of wild type and Delta strains with or without the T492I substitution, that mutated strains accumulate quantitatively more mutations in other genes, thus generating variants similar to the circulating omicron strains. I have no further remarks for the co-authors. This is a robust manuscript that demonstrates the existence and importance of mutation-driven predisposition in viral evolution for SARS-CoV-2 and potentially opens the way to investigate such an important phenomenon in other viruses.

Response: We greatly appreciate your positive comments.

Responses to Reviewer 3

Thank you to the authors for addressing the concerns I have raised through their reviewed submission. The experiments are extensive and the data is of interest. The changes does drive a fitness gain BUT the virus will always will be the sum of many parts and will evolve to gain fitness under many varying conditions. The appearance of Omicron-like lineages in waste-water that pre-dates their appearance, supports replication and evolution of Omicron lineages to a very specific tissue environment (intestine). Yes this change may accelerate the virus to get to that point and importantly represents a fitness gain alongside many other changes in the virus that have enabled to persist in the face of high level mature immune responses. The results herein are indeed of interest but are at times oversold to be the driving force of major evolutionary steps in the virus. The mechanism of accelerated evolution through the interaction with cytidine deaminases is also interesting but not well developed. Cytidine deaminases are observed to significantly restrict viral replication through hypermutation (well at least for HIV). So how does the mechanism change here? and if cytidine deaminases were removed from cells, would this render the polymorphism redundant? I'm not requesting the authors do additional experiments, but rather qualify that far more mechanistic work would need to be done to make this claim.

Response: Thank you very much for your comments and insightful suggestions to improve our manuscript. Following these suggestions, we revised the sentences relevant to the discussion of the driving force and the appearance of Omicron-like lineages in the waste water in lines 534-537, 539-542 and 548-553, as shown below.

“Aside from the predisposition effect of T492I, the host/environmental factors (such as herd immunity) may be an important force that shapes the viral evolution, since the host/environmental factors induce selection on the mutation-driven phenotypes.”

“The driver mutation and the host/environmental factors may jointly and interactively regulate the evolution of the SARS-CoV-2. The evolution of SARS-CoV-2 may also be the sum of many parts, resulting in the gain fitness under many varying conditions.”

“The appearance of Omicron-like lineages in the waste water, predating the emergence of Omicron variants, supports the capability of Omicron lineages to replicate and evolve in a very specific tissue environment (e.g. the intestine)[86], suggesting a potential fitness gain possibly accelerated by T492I and other adaptive mutations to resist a high level of mature immune responses.”

We also have included the qualify that far more mechanistic work is needed to understand the changes in the mechanism of deaminases to restrict viral replication through hypermutation and whether the removal of deaminases from cells may render the polymorphism redundant., in lines 490-493 of revised manuscript.