nature portfolio

Peer Review File

Bat genomes illuminate adaptations to viral tolerance and disease resistance

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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:

Referee #1

(Remarks to the Author)
This manuscript presents 10 new assemblies of various bat species to decipher the immune system adaptation across the bat branches. All the newly assembled 10 bat genomes are in high quality, potentially suitable to serve as a reference genome for gene-based analysis as such the authors performed. The authors performed a comparative analysis comprising 115 mammalian species and 228 phylogenetic branches. Personally, I was amused reading the article, even though my background in immunology is very limited. This work is an exemplary landmark for why having high quality reference assemblies across different species is such important. The results seem to be compelling, although some of the details needs to be addressed further.

What is the chance the immune gene on the other haplotype is playing an important role? Given that immune genes are highly diverged, the chance for assembling them is very high. It might be worth looking into the alternate haplotype assembly for the gene pairs and see if the results are still concordant.

Are the selected immune genes structurally different? How are they located (differently) in the genome? I was surprised that nothing is mentioned about structural differences, which I was expecting to see at least in a comparison among the long-read based bat assemblies given their longer contiguity.

Supplementary Table 3 and Supplementary Table 5 is missing a lot of the assembly accessions used this study. Also, I think it is fair to properly cite the related publications for each genome assembly used in this study to credit their work.

How was the QV estimated? If HiFi reads were used for the 9 genomes with no short-read data (10X), it should be discussed that the QV could be inflated, and remaining base issues (e.g. homopolymer or microsatellite repeat errors) that are systematically prevalent in HiFi reads are undetectable.

Which basecaller version was used for the 81X Megaderma sppasma ONT reads? What was the N50 of the read length?

The “BUSCO - proteins from TOGA annotation” in Supplementary Table 2 shows high duplication rate (40-41%) compared to the “BUSCO - genome (human as reference)”. Why is this the case? Some explanation would be beneficial to understand the background.

The Github repo for sharing codes has no information on the genome assembly, scaffolding, curation, polishing and validation. The community will benefit from sharing exact parameters used for establishing and assessing the assemblies.

Referee #2

(Remarks to the Author)
This is a very interesting paper describing 10 new very high-quality genome assemblies for 10 bat species, and exploring selection on immune related pathways in the bat lineage compared to other mammals. The results are novel and significant, but I have concerns about the clarity of the manuscript and the rigor of some of the immune related analyses. I think there may be too much focus on the sole hypothesis of selection on viral tolerance without consideration of other explanations.

Overall, this is a paper of wide interest and deserving of publication in Nature.

I was also disappointed that while the manuscript observes variation in selection between bat lineages, it doesn't explore that any further. For example, in figure 2C, are bat species with more immune process genes under selection also the bat species that are unusually long lived or are hibernators vs the ones with fewer immune process genes under selection? Is there any correlation with the viral diversity discovered in those lineages?

Specific comments on the text:
The introduction includes analyses done for this paper. Specifically, the analysis of viruses detected in bats vs on mammals, and in different bat families (presented in figure 1A and 1B). If the analysis of prevalence is new, it should be moved to the results. I'm also not clear which of the comparisons is statistically significant and how that determination was made. Are the number of coronaviruses detected in bats significantly higher given that there may be a bias towards testing for viruses in bats? The text refers to # individuals, not # of species or # different populations, but presumably individuals within a species or population are not entirely independent data points. There needs to be more details on the significance of these comparisons.

The text assumes more familiarity with bat taxonomy and ecology than most readers will have. It would be more interesting with more information on the animals. For example, the authors write that they included four rhinolophid species and three hipposiderid species, but don't describe what these names refer to, or why sequencing species from both would be an important thing to do. Based on the latin names, one is a genus and one is not. There is no information on the overall number of bat genera or species or whether the species selection represents a broad selection across the order or a specific focus on particular lineages, or exactly why these 10 species were selected.

I have no concerns about the genome sequencing and assembly. The genomes are exceptional. I am intrigued by the comment that most of the samples came from museum samples, and I think that deserves more focus in the main text. It has major implications for the field of genomics. What type of museum samples? What was the DNA quality? Was it of similar quality / molecular weight as from fresh samples, and did it vary depending on the type of museum sample? Were the genomes made from museum samples comparable to the other genomes? Does this show that museum samples are going to be broadly usable for producing high quality genomes?

Figure 1C & 1D needs to be revised to be interpretable by someone with poor color vision - the colors are not easily distinguishable. In Figure 1E, I'm not sure what the interpretation should be of genes with "inactivating" mutations. Are the authors implying that these genes are not functional/lost in many bat species, or that this is most likely a reflection of genome assembly quality? It would be good to clarify that, and whether the high numbers of inactivated genes are surprising.

After the description of the new genomes, however, the manuscript gets a bit confusing. The title of the paper implies that the authors were able to detect adaptations for immune tolerance because the new genomes are "reference quality". In the manuscript, there doesn't seem to be a clear justification of this. To detect selection, the authors used genomes for 20 bat genomes and 95 species from other mammalian orders, but it is not clear whether the fact that the new bat genomes are high quality mattered for the results. If the analysis had been done with lower quality genomes, would the findings have been any different?

I appreciated the section titled "Selection in immune genes is most prevalent in bats" which assessed whether the enrichment for selection at immune-related genes in bats was exceptional compared to other mammalian orders. However, the lack of numbers was concerning. The main text should include some indication of which results are significant after correction and p-values, but this is missing from both the text and figure 3. For the heatmap in figure 2 A, D and E, I'm not sure which results / boxes are significant, and whether NA means that no genes overlapped or the enrichment was not significant.
The bats really don't look all that different from the rodents in figure 2A. How meaningful is it that bats have the "strongest enrichment" or is it more that bats and rodents have the strongest enrichment and then there is everyone else?

While the manuscript focuses on immune genes, the enrichment for Response to stimulus and other processes seems stronger. Are these enrichments also driven by immune related genes? It would be helpful to have the equivalent of figure 2C and 2D for other biological processes with significant enrichments and information on whether the selection for those processes is similarly timed.

Figure S8 is supposed to show all child terms of high-level GO terms in Figure 2. All of them appear to be immune system related, though - how were the terms chosen for inclusion in this figure?

The authors state "Together, this suggests that immune system changes originated early in the chiropteran lineage and coincided with the evolution of powered flight." This implies causality which is not supported by data, and it needs more context - flight hasn't been mentioned since the introduction. Are there other traits that evolved at the same time that would also be explanations?

The section titled "Relevant immune-related changes in bats" is very problematic and needs extensive revision. Right now, it feels like the authors are cherry picking their way through a series of vignettes chosen to fit their overarching point that immune genes are under selection (they write "we intersected knowledge about genes involved in immune responses to corona- and other viruses with positively selected genes"). If that is all this is, it should be relegated to the discussion. It's not clear how they chose which genes to discuss, or whether there is more selection on coronavirus response genes in bats

than in other orders. I can't figure out how many strongly selected genes they are not discussing because they can't make an immune related story about them. If their argument is that specific immune response pathways are under selection, there should be some kind of analysis showing that that pathway has an excessive number of selected genes. It's also not always clear what information is based on previous studies and what statements are related to observations of the data in this paper. I think most of the text is discussing previous work, and not the results from this paper, which should be the focus.

The section on "ISG15 of rhinolophid and hipposiderid bats vary in antiviral activity" is really intriguing, and their results are a beautiful demonstration of how complicated it is to connect a particular change to a particular phenotype (even an amino acid change) when you are working in an evolved and complex system. I think it would be valuable if that aspect of the results were given more prominence. The major missing component, though, is a justification for why this particular mutation in this particular gene was chosen for functional investigation, given the many different immune genes described in the previous section. It would be helpful to get more information on that.

The ISG15 section also was not very readable and had too many acronyms. It might help if each paragraph started with a sentence giving the big finding / main point before delving into the methods.

Discussion
The statement "However, compared to other mammalian orders, bats exhibit the strongest enrichments for immune gene selection, providing genomic evidence that bats possess unique immune system adaptations." is too strong. An enrichment for selection at immune genes alone is not evidence of unique adaptations.

"This supports that the evolution of powered flight and immune system adaptations are linked" - is powered flight the only thing different about Chiroptera? Is it really evidence for anything more that cooccurence? More support/context for this statement would be helpful.

"While we applied the regression model specifically to immune genes, this approach could be a generally-applicable strategy to reveal lineages (branches) having an excess of selection on genes belonging to particular functional categories." I think (unless the regression model is very arduous to apply) it would be reasonable for a paper of this scope to do this analysis of other functional categories as well.

Referee #3

(Remarks to the Author)
Bats, known as natural reservoirs for viruses, have been found to survive many viruses that are highly lethal to humans, such as SARS-CoV, SARS-CoV-2, MERS-CoV, Marburg, and henipaviruses. Several studies have shown that bats have significant functional differences in their innate and adaptive immune systems compared to other mammals, enhancing their antiviral immune response and reducing inflammatory signaling. However, the genetic and molecular mechanisms responsible for bat resistance to viral disease remain largely unknown. Morales et al. sequenced high-quality genomes for ten bat species, including those that harbor coronaviruses, to investigate the genomic basis of disease resistance. Their analysis indicated that bats possess higher immune gene selection than other mammals, including genes linked to viral detection and entry, inflammation regulation, antiviral mechanisms. They found that rhinolophid and hipposiderid bats display a deletion of a cysteine residue critical for ISG15 homodimer formation. The authors propose that this cysteine deletion in the bat ISG15 protein results in increased intracellular protein conjugation and decreased secretion of ISG15 into the extracellular space, thereby limiting the extracellular pro-inflammatory role of ISG15.

The authors have successfully built high-quality genomes for 10 bat species using long-read and long-range sequencing technologies. Compared to low-coverage or short-read NGS technologies, their approach significantly improves the contiguity of genome assemblies and enables better resolution of repetitive genome loci, where important immune gene loci reside. The resulting assembly and annotation, along with accompanying genomes, are of exceptional quality. Furthermore, the authors conducted a genome-wide screen and analyzed orthologous genes that have undergone positive selection across various mammalian orders, providing strong genetic evidence of positive selection of immune-related genes in bats. Overall, this aspect of the study is expected to provide a highly valuable asset for investigating the evolutionary history and genetic underpinnings of bat biology and adaptations.

The major weakness of the study relates to the data on the proposed antiviral mechanism of bat ISG15, which do not convincingly support the conclusion that rhinolophid and hipposiderid bat ISG15 with a cysteine deletion remains intracellular and exhibits increased ISGylation to dampen aggressive inflammatory responses. It is unclear why ISG15 in Rhinolophidae and Hipposideridae did not show a consistent antiviral difference when compared to human ISG15, although the authors propose that this may be due to additional mutations in individual bats. Moreover, the data on the expression and conjugation of overexpressed bat ISG15 in human cells are not convincing, and there is a concern about antibody compatibility in detecting bat ISG15. These data require extensive additional controls, and also confirmation experiments in bat cells, such as whether restoring Cys78 of bat ISG15 alters its ISGylation and secretion capacity in bat cells (see also specific comments below).

Major points:

1.As shown in Figure 4A, some bats still retain the cysteine residue (Cys78 in human). To validate whether this cysteine is indeed essential for bat ISG15's antiviral function and its ability to limit pro-inflammatory responses, the authors should also include one representative bat ISG15 which contains Cys78 in the experiments in Figures 4B-4H.

2.Figure 4E. It is somewhat puzzling that, despite having a Cys78 deletion like most other bat ISG15s, the ISG15 of Aselliscus stoliczkanus and Rhinolophus trifoliatus do not show an increased antiviral activity compared to human ISG15 against SARS-CoV-2. However, Rhinnolophus yonghoiseni, which is closely related to Rhinolophus trifoliatus, exhibits a significantly enhanced antiviral activity. Does this mean that the mutations between these two bat ISG15 proteins account for their antiviral function against SARS-CoV-2? It is suggested to look at the specific sequence differences and conduct mutagenesis to explain this phenomenon.

3.Related to line 427-430: Since the Cys78 of ISG15 is absent in rhinolophid and hipposiderid bats, why not delete Cys78 in human ISG15 rather than mutating it to validate the function of Cys78? In Figure 4G, restoration of this cysteine in R.affinis ISG15 did not affect its antiviral effect, suggesting that Cys78 may not be important for the antiviral function of bat ISG15. In contrast, mutating Cys78 in human ISG15 significantly reduced SARS-CoV2 replication. Why is this?

4.In Supplementary Figure 19-22, the range of topologically distinct conformations predicted by AlphaFold2 does not necessarily indicate an unstable structure, but rather reflects the potential flexibility and adaptability of a protein under different conditions. To determine if homodimers of bat ISG15 proteins with Cys78 deletion differ from human ISG15, additional evidence is needed, such as ISG15 dimer detection by SDS-PAGE of both bat and human ISG15 in non-reducing conditions (without 2-Mercaptoethanol) (like in PMID 18606809).

5.Why are the titers of Homo sapiens-C78S and Rhinolophus affinis-S77-78C missing in Figure 4G? Please include these.

6.To exclude the effects of endogenous human ISG15, the authors should conduct experiments overexpressing bat ISG15 in ISG15 knockout HEK293 or A549 cells. Additionally, it is currently unknown whether the ISG15 antibody used in this study is effective in detecting bat ISG15. For example, the ISG15 blots in Supplementary Figure 27 do not show any apparent differences between the transfection of empty vector and bat ISG15, indicating that either the overexpressed ISG15 levels are very low or the antibody is ineffective. This is a major concern. Given the uncertain expression levels of bat ISG15 (compared to human ISG15), the related antiviral functional assays against viruses and pro-inflammatory functions of bats ISG15 may not be entirely convincing.

7.Figure 4H: It is somewhat hard to believe that there is no free ISG15 in all bats. As mentioned above, there is a major concern about antibody compatibility. In addition, the authors need to confirm whether the human ISGylation machinery works for bat ISG15. Therefore, the authors should compare the bat ISG15 conjugation capacity in ISG15 knockout background. In these assays, bat ISG15-AA mutant (LRGG mutated to LRAA), which cannot be conjugate to substrates, should be included as a negative control.

8.Given the potential differences in the ISGylation machinery between bats and humans, the authors should express in bat cells two representative bat ISG15 proteins with Cys78 deletion, and also validate whether restoring Cys78 can alter their ISGylation and secretion capacity.

9.In the experiments shown in Figure 4H (right blots), wildtype ISG15 of human and Raff should be included as well. Otherwise, the secreted ISG15 levels of the mutants and wildtype ISG15 on different blots cannot be compared.

10.Ubiquitin-specific protease 18 (USP18) is the primary ISG15-specific protease that counteracts ISG15 conjugation. Does it undergo positive selection in bats? Since the conjugation of overexpressed bat ISG15 in human cells may differ from that in bat cells due to differences in human and bat USP18, it is suggested to synthesize a representative bat USP18 and check if it functions normally in deISGylating bat ISG15 when compared to human USP18.

11.In lines 417-418, the authors state that the viral N protein was significantly reduced by human and R. ferrumequinum ISG15 overexpression. However, in Figure 4H, no obvious difference in HCoV-229E N protein was shown when human and bat ISG15 were overexpressed. Why is this?

Minor points:
1.Supplementary Figure 27 and Supplementary Figure 30: These blots are of low quality and should be repeated and improved.
2.Molecular weight markers should be included in all blots.
3.In Supplementary Figure 9, “(A)” and “(B)” are missing from the figure.
4.In Supplementary Figure 10, “(B-I)” should be changed to “(B-E)”.

Referee #4

(Remarks to the Author)

In their manuscript, Morales and Dong et al. present new genome assemblies of ten bat species and, by analysis of positive selection across these genomes and those of other mammalian species, conclude that the positive selection in the immune genes is highest in bats. They review the current knowledge about the immune roles of the selected genes that may help the reader interpret the findings. Then, they analyze in detail one of the genes, ISG15, in which a cysteine 78 residue is deleted in some of the bats. They note that in other species, Cys78 "is required for the formation of stable ISG15 homodimers and its extracellular cytokine function", and that "mutating Cys78 enhances ISGylation, likely because dimerized ISG15 is not usable for ISGylation". They thus start the ISG15 analysis with structural modeling using AlphaFold, I-TASSER, and molecular dynamics (MD) to conclude that even though AlphaFold predicts dimeric structures for bat ISG15s, the dimers are unstable, compared to the stable human ISG15 AlphaFold dimeric model. They follow by an experimental analysis in cell culture to investigate the capacities of bat ISG15s to restrict infection of four different viruses. They find that different ISG15s have various effects on restricting the viruses, but the results depend on the bat and virus species, so other factors must also play a role. They finish by confirming that bat ISG15s that lack Cys78 are not secreted from the cells infected with coronavirus as is the human Cys78-containing ISG15.

Overall, the manuscript is an exciting and accessible read, even for a general audience from other fields. The finding that immune genes are positively selected in bats and the presented specific list of the genes addresses the general curiosity about mechanisms behind bats being such a successful reservoir of various viruses. However, focusing my revision on the structural modeling part of the manuscript, I have major comments about this part, which in its current form does not support the conclusion that the human ISG15 dimer is stable while the non-cysteine bat variants are unstable:

1) The confidence scores of the AlphaFold models, especially those shown in Supp. Figures, should be shown. These should include at least the global pTM, ipTM, the per-residue pLDDT (mapped to the models using the standard AlphaFold pLDDT coloring scheme), and, for the dimeric models, the PAE plots.
2) Are the AlphaFold interaction scores (ipTM, PAE) high for the bat dimers? If so, how the claimed instability of MD simulations could be explained? Do the authors mean that the AlphaFold dimers false positives in these cases? This should be explained to the readers.
3) I do not understand why the I-TASSER model was used to predict the structure of the human ISG15 monomer (Supp. Fig. 22), as crystal structures seem to be available (PDB IDs: 1Z2M, 7S6P). The supp. fig. 22 should either include the panel comparing the I-TASSER model to the crystal structure (and have all-atom RMSD in the legend) and explain the reason for modeling to the reader or be redrawn to use the crystal structure.
4) If it is necessary to use a theoretical model of the human ISG15 monomer for some specific reasons, why use I-TASSER instead of the usually more accurate AlphaFold? The supp. fig. 22 should either include the panel comparing the I-TASSER to AlphaFold – as the current baseline modeling program – or be redrawn to use the AlphaFold model
5) The use of MD to validate AlphaFold models is very interesting. Has this approach been systematically validated yet, though? Do we actually know yet what a more accurate method for assessing the confidence of AlphaFold dimers in general is – AlphaFold native scores or stability in MD simulations?
6) The analysis of the MD simulations is limited to clustering MD conformations and showing pictures of cluster representatives. This makes it difficult to appreciate whether the simulations support the claim about the bat ISG15 dimers being unstable and human ISG15 dimer stable. In fact, for the bat examples (Supp. Figs. 19 and 20), the actual dimeric interface seems to be preserved in all representatives while only the outer non-interacting domains are flying around. On the other hand, for the human ISG15 (Supp. Fig. 20), even the dimeric interface seems to change, although it is hard to judge as the models are shown in different orientations (the disulfide bridge remains, but it is obvious as MD cannot break bonds). A quantitative analysis is absolutely necessary to show whether dimeric interfaces are more stable in the human ISG15, possibly including positional root mean square deviations, a fraction of native contacts from the initial model across the simulation, and overlays of the structures superposed on the dimerization interface across the simulation time. Perhaps the following publication could provide some guidelines on how to perform a convincing analysis:
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8444332/
7) Related to the above, using whole-dimer RMSD for the comparison and clustering is not informative for the questions asked about the stability of the interfaces – if non-interacting domains are very variable, the RMSD and global variability will be high even if the dimeric interface was preserved. The clusters will represent the variability of those other domains rather than the interface. The analysis should focus on the *interface* RMSD and interface contact analysis.
8) The quantitative analysis should also consider the AlphaFold confidence scores. For example, suppose the dimeric interface is composed of two parts, one well supported by the PAE and pLDDT scores and another poorly scored (as is frequent in AlphaFold models). In such case, it should be checked whether the confident part remains stable while only the non-confident one is variable (in other words, the interface RMSD or contact analysis including the non-confident part would give artificially high variability).
9) The quantitative analysis may reveal that the human dimers do not have more stable interfaces than bats except for the disulfide bridge. Still, as the disulfide bond cannot be broken in MD simulation, the human ISG15s remain bound. MD is not necessary to conclude that two proteins connected with a disulfide bond would be stable.
10) Na+ and Cl- ions were used only for neutralizing the system. Shouldn't the simulations be run in the usual closer-to-physiological 150 mM salt concentration? Could the reduced salt concentration affect the stability of the dimers in the simulations?
11) I find it odd that the protein distorts so much, but the dimer doesn't fall apart in the bat simulations. This could be either that the dimers are stable after all, the low salt issue over-stabilizing the interfaces, or too small bounding box. For the former two, see comments above, but for the latter, the authors should show a snapshot of the initial system, including lines indicating the simulation box to make sure the padding was big enough.
12) The equilibration time seems a bit short - 100 ps - while some studies report longer times ranging from a few nanoseconds to tens or even hundreds of nanoseconds (https://pubmed.ncbi.nlm.nih.gov/17280090/, https://onlinelibrary.wiley.com/doi/10.1002/prot.26161). Although the equilibration time length of time required to equilibrate

a protein system in molecular dynamics simulations can vary depending on the specific system being studied, the force field used, and the simulation conditions, could it be that the ISG15 models fall apart due to a lack of equilibration? Perhaps, in this case, a longer equilibration is needed because the starting structures are theoretical models?
13) Videos of representative simulations should be included in the manuscript supplementary information. They could perhaps show whether the models distort quickly due to the lack of equilibration.
14) The analysis of DDG of mutations in ISG15 using DynaMut doesn't seem to add much to the manuscript and brings additional prediction uncertainty (due to the non-perfect predictive power of the method and because the analysis does not consider the dimeric conformation), but if the authors prefer to keep it, it probably should be redone with the crystal structure rather than I-TASSER model.
15) The Methods section says simulations were run for 1 microsecond, while the text and figures state 3 microseconds. I guess this is just a typo in the methods section?
16) On page 7, lines 219-222 – "42 observed vs. 21 expected selected immune-related genes" in Chiroptera is mentioned as "a larger relative number" compared to "42 observed vs. 24 expected in Afrotheria" – this ratio is almost the same, is there a typo here?
17) The article already very nicely explains several basics of immunology or explains the modeling methods, making this manuscript accessible to a general reader. Could you also explain the method for detecting positive selection behind the aBSREL software? The "an adaptive branch-site random effects likelihood method implemented in HYPHY" is not clear to a general reader.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
The authors fully addressed all my comments and concerns. I thank the authors for their detailed, thorough response.

Referee #2

(Remarks to the Author)
This is a revised version of a previous manuscript. The authors have addressed all my questions clearly, and I appreciate the thoughtfulness of their response. This is an elegant and well written paper that delves into the complexity of evolution in response to pathogens across the mammalian class, and deeply within the bat lineage. It illustrates the power including high quality reference genomes as part of a much larger resource of both higher and lower quality genomes to explore evolutionary questions. I have no further critiques and recommend it (enthusiastically!) for publication.

Referee #3

(Remarks to the Author)
The authors have performed extensive additional experiments to address this reviewer's previous concerns; however, some of the biochemical/mechanistic data are not of sufficient quality and therefore fail to support the major conclusions about the role of deletion of Cys78 in ISG15 in antiviral activity. Therefore, the major concerns about the molecular mechanism presented in Fig. 4 and 5 still remain.

Specific major concerns:

1. In Figure 4B, the expression level of R.a ISG15 in lane 5 is significantly lower compared to that of other species or mutant, making it difficult to accurately compare their antiviral abilities in Figures 4D and 4E.

2. The new results presented in Figure 4 indicate species- and virus-specific differences in antiviral activity that are not solely explained by the presence or absence of Cys78. This suggests that additional mutations in individual bats (as shown in Figure 4A) may also influence ISG15's antiviral activity. Therefore, it is unconvincing that the differences in antiviral ability are primarily due to the Cys78 deletion.

3. There are still major concerns about the assays showing ISGylation in the revised manuscript (SI 38 and 39). Overexpression of ISG15 in HEK293T cells alone is insufficient to detect robust ISGylation due to the absence of the complete ISGylation machinery. How could the ISGylation bands predominantly appear between 17-35 kDa? In SI39, the smears observed in lanes 7-14, with or without PLpro, likely reflect free ISG15 expression. Overall, the proposed ISGylation bands appear to be non-specific or possibly due to gel running issues.
Furthermore, why does lane 6, which overexpresses PLpro, show an enhanced smear compared to lane 5? Consequently, the quantitative data on conjugated ISG15 in Fig. 4 is not convincing and the overall proposed mechanism not sufficiently supported by the new data.

Referee #4

(Remarks to the Author)

In the revised version, the figures and plots supporting the AlphaFold modeling and molecular dynamics better illustrate the confidence in the models and the impact of equilibration length and salt concentration on the simulations. The confusing I-TASSER model and DynaMut analysis have been removed from the manuscript. While AlphaFold models of different dimers are presented, they are deemed unreliable as their confidence levels do not align with the experimental data. A noted contradiction is that the human dimer, expected to be stable, has very poor interface scores. Conversely, the R. sinicus dimer shows generally good scores despite expected instability. The authors' interpretations seem plausible, as the local scores at the interface of the bat model are lower than in other parts of the structure, and it remains unclear whether AlphaFold scores correlate with stability. The molecular dynamics simulation presentation has improved, displaying data on equilibration and salt concentration, following my suggestions.

However, in my opinion, the molecular dynamics simulation still does not convincingly show that bat ISG15 is unstable:

- The two subunits of the bat ISG15 appear to remain attached throughout the simulation, as evidenced by the improved Figures 28 and 29 and the supplementary videos. The interaction interface also seems to be preserved throughout the simulation trajectories. In the review attachment, I provided snapshots from the trajectories for both the human and R. sinicus dimers, which demonstrate the stability of the interfaces for the human dimer (beyond the disulfide bond) but also of the bat dimer interface. The interface, encircled in yellow in the attachment, appears stable and maintains a similar conformation across all snapshots and all three trajectories, or at least is not less stable than in the human simulations. In my opinion, the interface is stable in the molecular dynamics simulation based on this data. This aspect was overlooked in the quantitative evaluation below, likely because the evaluation focused only on hydrogen bonds, as discussed below.
- To provide a quantitative assessment of dimer stability in the simulations, the authors stated: “Similar to the procedure described in (Jandova et al. 2021), we analyzed the fraction of native hydrogen bonds between the two monomers over the course of the simulation.” However, Jandova et al. did not primarily use the fraction of hydrogen bonds, but the fraction of native contacts, calculated with the “gmx hbond-contact” tool, where I believe they meant the gmx hbond program with the -contact option, which focuses on contacts within the cutoff distance rather than hydrogen bonds. Jandova et a. define the fraction of native contacts as “Fnat is the fraction of native contacts that takes into account any atom pair-forming contacts between proteins within a 5 Å distance cutoff”, following the standard in the field. Jandova et al. even noted that “the number of hydrogen bonds shows the smallest importance” in their machine learning classifier of native and non-native interfaces.
- The authors of this manuscript observed very few hydrogen bonds at the interface of the bat dimer but nevertheless then used the fraction of native hydrogen bonds to monitor the stability of the dimer. If the interaction in the model is not mediated by hydrogen bonds, what is the point of using this as a criterion for monitoring stability? Perhaps the interface is more mediated by hydrophobic interactions instead? Monitoring the fraction of native contacts would be more appropriate and independent of the interaction type. Such analysis is necessary minimum to show whether the bat dimers are less stable that the human dimer.

For the models of PLpro with ISG15, which were added in the revision, only fragmentary data is provided, that does not allow assessing whether the models are good enough for the conclusions about the interfaces:

- No pLDDT or iPTM scores are provided.
- The PAE plots in Supplementary Figure 41 use a different coloring scheme than Supplementary Figure 24, and the color legend is not provided.
- The legend of Supplementary Figure 41 does not clarify what “differential paired alignments” means. I initially thought the authors meant only regions that differ, but not all regions like those highlighted, so clarification is needed.
- There are no figures with the models, just videos, making it very hard to compare the models side by side.
- The authors mention “we utilized the recently identified crystal structure” for modeling—does this mean they used the structure as a custom template in ColabFold?
- The authors state: “The human ISG15:PLpro model fits the known structure of the complex well (Supplementary Figure 41)”, but that figure does not contain any comparison to the known structure, just PAE plots—a figure comparing the structure and the model should be included.
- The interfaces discussed on pages 15-16 should be shown in a figure, along with pLDDT coloring, and related to the PAE plots. If the interfaces are not similar to the human structure and exhibit low pLDDT, they should be approached with caution and mentioned as such in the text—in my experience, it is possible to obtain false positive AlphaFold models of interactions, and they often have good PAE but poorer interface pLDDT (as also suggested by the authors for the R.sinicus ISG15 dimer).

Version 2:

Reviewer comments:

Referee #3

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

Referee #4

(Remarks to the Author)
The revised manuscript demonstrates a proper re-analysis of native contacts, which is appreciated. While the figures still suggest that the subunits remain attached at the same interface as in the starting structure (marked by yellow circles in my

previous feedback), the quantitative analysis of the fraction of native contacts clearly indicates a loss of most of the contacts in the initial structure.

The PCA analysis is a useful addition, as it demonstrates the exploration of alternative states. However, it does not address whether the remaining few contacts differ across these conformational states. As mentioned earlier, the subunits appear to stay attached at the small interface I previously highlighted. Nevertheless, it is possible that these remaining contacts are quite loose, as indicated by the fraction of native contacts analysis, and could potentially break during longer simulations, as the authors suggest. The revised analysis provides readers with means to better assess this aspect, and since the computational analysis is not central to the manuscript, rather an indication complementing the experiments, I would suggest accepting this state.

The AlphaFold confidence analysis for the new models, previously incomplete, has been extended and now provides readers with a better tool to evaluate the models. While some discrepancies remain between model confidences and conclusions, it is acknowledged that the models are not critical for the main conclusions, which are primarily based on experimental results. The current presentation of these models is therefore acceptable.

Point by point response

Referee #1 genomics, genome assembly

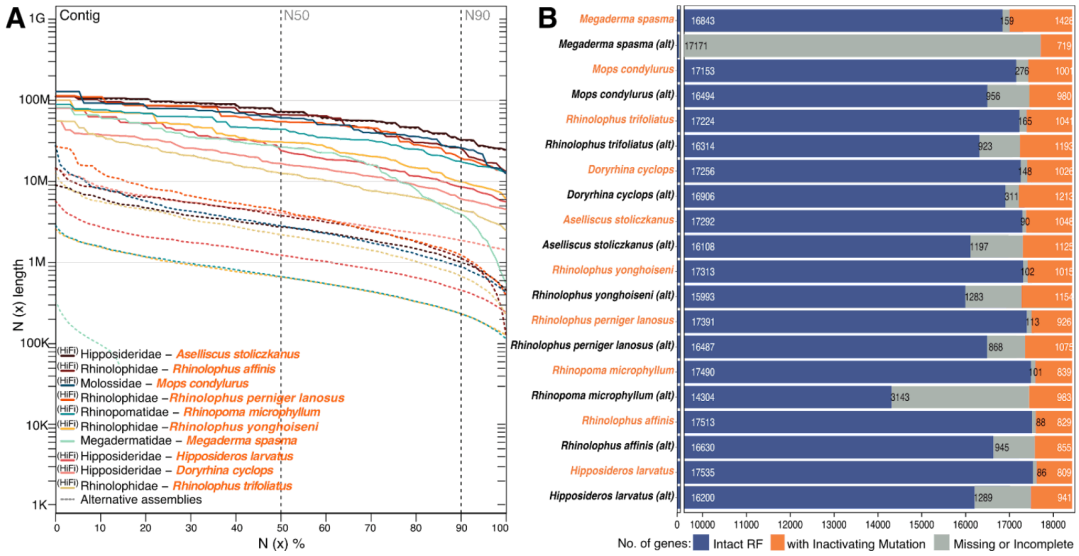
This manuscript presents 10 new assemblies of various bat species to decipher the immune system adaptation across the bat branches. All the newly assembled 10 bat genomes are in high quality, potentially suitable to serve as a reference genome for gene-based analysis as such the authors performed. The authors performed a comparative analysis comprising 115 mammalian species and 228 phylogenetic branches. Personally, I was amused reading the article, even though my background in immunology is very limited. This work is an exemplary landmark for why having high quality reference assemblies across different species is such important. The results seem to be compelling, although some of the details needs to be addressed further.

We thank the reviewer for these kind words and the insightful comments that helped to improve our manuscript.

1) What is the chance the immune gene on the other haplotype is playing an important role? Given that immune genes are highly diverged, the chance for assembling them is very high. It might be worth looking into the alternate haplotype assembly for the gene pairs and see if the results are still concordant.

We thank the reviewer for raising this point, as we have not analyzed the alternative haplotype assemblies so far. To address this point, we now repeat-masked the alternative haplotypes using the repeat library inferred from the primary haplotype, aligned the alternative haplotypes to human, and applied TOGA (the statistics are in the new Supplementary Figures 5 and 12 and in Supplementary Table 2). As expected from the HiFiasm procedure that produces a primary assembly with the highest completeness and an alternative assembly consisting of haplotigs in heterozygous genomic regions, the alternative assemblies are less contiguous than the primary assemblies. Nevertheless, their contig N50 values range from 0.60-4.36 Mb (excluding *Megaderma spasma,* which was not sequenced using HiFi) in comparison with the primary assemblies whose values are 12.5-72.2 Mb.

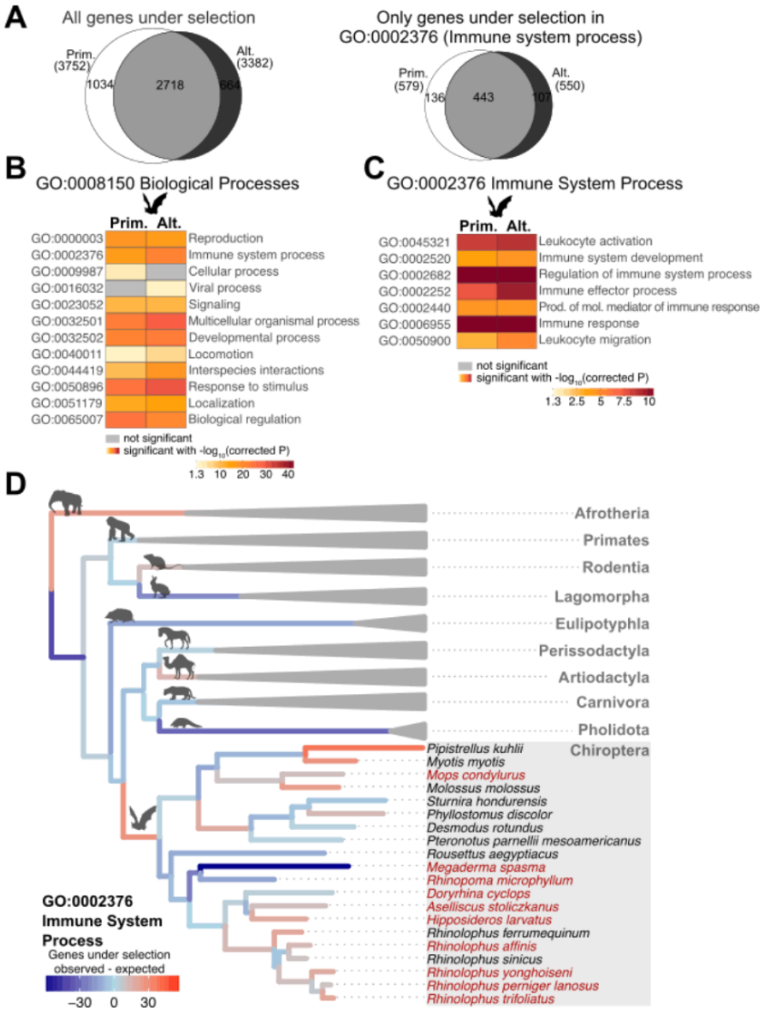
We also compared the status of 18,430 ancestral placental mammal coding genes, as inferred by TOGA. As expected, the alternative assemblies have more genes that either contain missing sequences or are completely missing, as genes in homozygous regions would not be incorporated into the alternative assemblies. Nevertheless, most HiFi-based alternative assemblies have about 16,000 genes that are fully present and have an intact reading frame (dark blue in the figure), which is a higher number compared to short read-based assemblies (Supplementary Figure 4). The alternative assembly of *Megaderma spasma*, which was assembled from reads generated by Oxford Nanopore Technologies, using Canu v2.2 in -nanopore mode, has very few intact genes, likely because the contig N50 is only 125 kb and the alternative assembly comprises only 311 Mb (Supplementary Figures 5, Supplementary Tables 2).



Supplementary Figure 5. Comparison of primary vs. alternative bat assemblies.
For our 10 new bat genomes, we compared contiguity (A) and status of 18,430 ancestral mammalian genes inferred with TOGA (B) between the primary assembly (solid lines in A, orange in B) and their respective alternative assemblies (dashed lines in A, black in B). As expected, alternative assemblies are less contiguous. Nevertheless, many alternative assemblies have more than 16,000 intact genes. *Megaderma spasma* was sequenced using ONT data and only has a 311 Mb alternative assembly.

As suggested by the reviewer, we investigated whether a selection screen produces concordant results when using gene sequences from the alternative assemblies. To this end, we replaced the 10 primary bat assemblies by their alternative assemblies, keeping all other 105 mammalian genomes, and repeated the key analyses. As shown in a new Supplementary Figure 12, we obtained similar results when using alternative assemblies, indicating that the key findings presented in our manuscript are robust to intra-species divergence that is partially captured in the alternative assemblies. We added to the main text:

“This pattern also holds true when using the alternative, instead of the primary assembly, for the ten new bat genomes that capture intraspecific differences (Supplementary Figure 12).”



Supplementary Figure 12. Enrichment results for genes under selection in bats and GO terms for biological processes and immune system process, comparing values when primary or alternative genome assemblies are used on selection screen.

We investigated whether a selection screen produces concordant results when using gene sequences from the alternative assemblies. To this end, we replaced the 10 primary bat assemblies by their alternative assemblies, keeping all other 105 mammalian genomes.

We then re-ran the genome-wide screen for positive selection, performed the same gene set enrichment analyses and explored the number of selected genes per branch. Since the gene sequences of non-Chiroptera species have not changed, we show only enrichments for Chiroptera, showing the values from Figure 2 that were obtained with the primary assemblies again for the purpose of a side by side comparison.

(A) Venn diagrams show the number of genes under selection when using primary vs. alternative bat assemblies. Importantly, the majority of the genes is under selection in Chiroptera irrespective of whether the primary or alternative assemblies are used. However, using primary assemblies, we found more genes under selection, likely because alternative assemblies do not contain all genes, as shown in Supplementary Figure 5.

(B, C) Heatmaps show enrichments of high-level GO terms (B) and child terms of “immune system process” (C). Enrichments are highly similar when using the primary or alternative bat assemblies, corroborating that immune gene selection is most prevalent in bats. In particular, bats have the strongest enrichment for “immune system process”, also when using the alternative assemblies. Some terms like “Immune effector process” (GO:0002252) are more enriched when using the alternative bat assemblies (158 of 2865 selected genes are annotated with GO:0002252) compared to using the primary assemblies (165 of 3207 selected genes are annotated with GO:0002252); this is because a higher proportion of selected genes is annotated with the GO term (5.51% for alternative vs. 5.14% for primary assemblies) despite fewer genes are under selection (158 vs. 165).

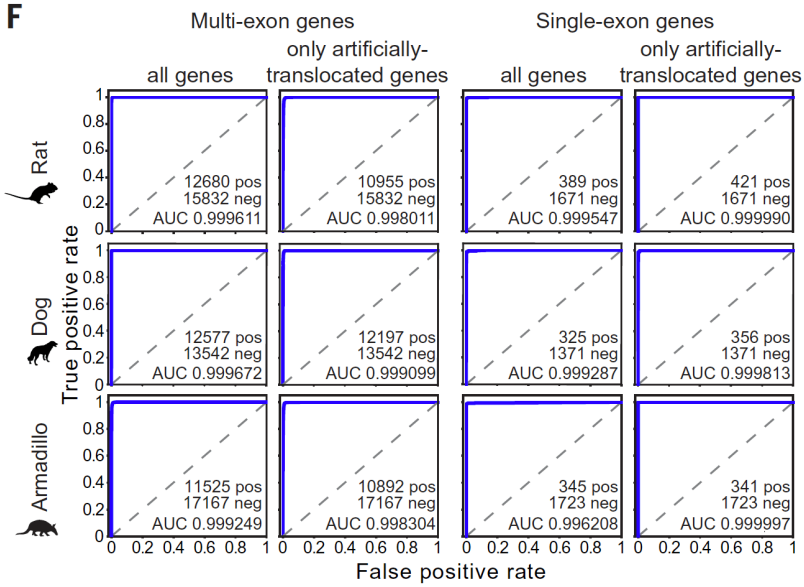
(D) As for primary assemblies (Figure 2C), the ancestral bat branch has more immune genes under selection than expected based on its length when using alternative assemblies.

Together, this analysis further supports that the key results of our screen are robust and not affected by intra-species divergence that is partially captured in the alternative assemblies.

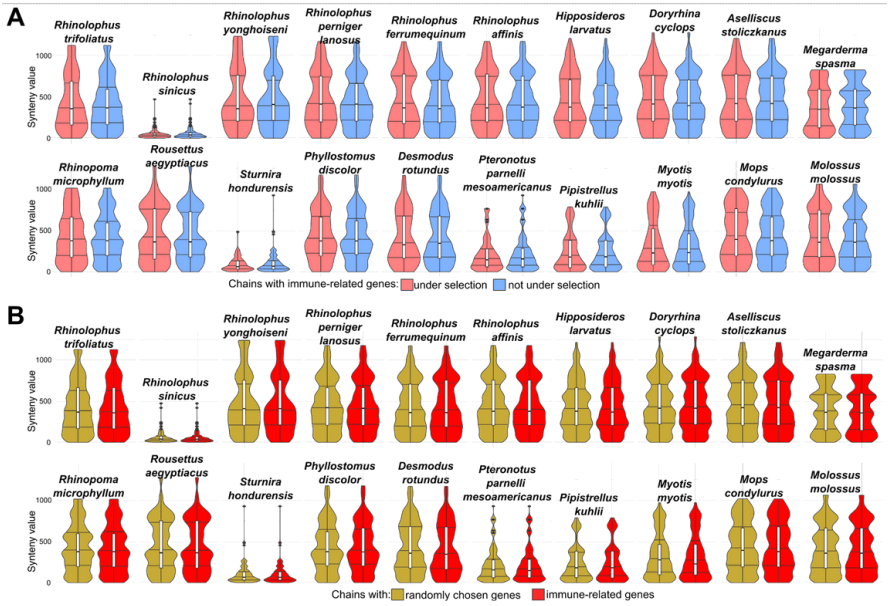
2) Are the selected immune genes structurally different? How are they located (differently) in the genome? I was surprised that nothing is mentioned about structural differences, which I was expecting to see at least in a comparison among the long-read based bat assemblies given their longer contiguity.

We agree that these are interesting questions, which we have not explored so far. Structural differences "inside" immune genes (such as exon deletions) will give rise to two distinct haplotypes, where one will be represented in the alternative assembly. Since we found highly similar results when using genes from the alternative assembly (point 1 above), we focused our analysis on structural rearrangements that could affect immune genes more often than other genes. If selected immune genes are more prone to undergo structural genome rearrangements (which will be visible in our chromosome-level bat assemblies) than non-selected immune genes, we expect the former to have lower synteny values. Please note that we use the term synteny here to refer to conserved gene order.

To this end, we made use of TOGA's ability to also accurately detect orthologs of genes that underwent genomic rearrangements such as inversions or translocations. We previously showed in Figure 1F in the TOGA manuscript (https://www.science.org/doi/full/10.1126/science.abn3107) that orthologous loci of rearranged genes that lack any synteny (column 2 and 4) are detected almost as well as orthologous loci of genes that are located in a context of conserved gene order.



As shown in a new Supplementary Figure 13A, we compared for each bat species synteny of immune-related genes that are under positive selection in bats and the remaining immune-related genes that are not selected in bats. This analysis revealed very similar distributions. We added to the main text: "Immune-related genes that are under positive selection in bats are not more prone to undergo structural rearrangements in bats than not-selected immune genes (Supplementary Figure 13)."
We also compared synteny between all immune-related genes and the same number of randomly selected genes, which also revealed very similar distributions (Supplementary Figure 13B).



Supplementary Figure 13. Selected immune-related genes do not undergo rearrangements more frequently than other genes.

To explore whether structural rearrangements affect immune genes that are under positive selection in bats more often than other genes, we leveraged TOGA's ability to accurately detect orthologs of genes that underwent genomic rearrangements such as inversions or translocations [9]. If a group of genes is more prone to undergo structural genome rearrangements, we expect these genes to have lower synteny values. We use the term synteny here as conserved gene order, i.e. the number of genes that align in co-linearity in the same alignment chain. A translocation or inversion of a single gene results a synteny value of 1 for this gene.

(A) We divided the 2,821 "immune system process" (GO:0002376) genes into those that are and are not under selection in bats. For each bat species and each gene, we plotted the synteny value of the respective orthologous chain.

(B) As in (A) but comparing all "immune system process" genes to control genes, obtained by randomly sampling 2,821 genes from the 17,130 genes included in our screen.

White box plots show the median, first and third quartile values. Barplots below each violin plot represent the total number of chains. For all 20 bat species, the distributions of the synteny values are highly similar, indicating that selected immune-related genes or immune genes in general are not more prone to undergo structural rearrangements in bats. As expected, genomes with lower scaffold N50 values (e.g. *Rhinolophus sinicus* or *Sturnira hondurensis*) have generally lower synteny values, as alignment chains by definition cannot extend across scaffolds.

3) Supplementary Table 3 and Supplementary Table 5 is missing a lot of the assembly accessions used this study. Also, I think it is fair to properly cite the related publications for each genome assembly used in this study to credit their work.

We thank the reviewer for pointing this out. These tables already contained the accession numbers of assemblies we obtained from NCBI; however, they were missing the source of assemblies we downloaded from the DNAzoo website. The reason for selecting several DNAzoo assemblies, which are sometimes not available on NCBI, was that we tried to include the best assemblies available at the time of our original screen. We have now added the download URL and added DNAzoo as the assembly provider to Supplementary Tables 3 and 5.

We also acknowledge DNAzoo as the assembly provider with the (Dudchenko et al. 2017) citation in the Acknowledgements.

4) How was the QV estimated? If HiFi reads were used for the 9 genomes with no short-read data (10X), it should be discussed that the QV could be inflated, and remaining base issues (e.g. homopolymer or microsatellite repeat errors) that are systematically prevalent in HiFi reads are undetectable.

The reviewer is right that QVs were estimated using the HiFi reads and not an independent Illumina read set. Using the HiFi reads directly for QV estimation is now common practice, but we agree that it is important to point out that they are based on the same reads used for assembly. We therefore edited the respective main text sentence to
"Although these QV estimates are an upper bound as they are based on the HiFi reads used for assembly, these base accuracy value estimates are two orders of magnitude higher compared with previous PacBio CLR or Nanopore-based assemblies [30,31]."

And added a section to the methods on "Consensus Quality Metrics (QV)", and point that:
"... although we note that these values are an upper bound as the reads used for assembly were used to estimate the QV, rather than an independent Illumina read dataset. Therefore, it is possible that remaining errors in the assembly are also in the HiFi reads, which are known to have issues in homopolymer and other simple repeat regions [142,144]."

5) Which basecaller version was used for the 81X Megaderma spasma ONT reads? What was the N50 of the read length?

We thank the reviewer for pointing out that these details were missing. We have now added them to the methods section:
"Both libraries were loaded on a Promethion device using R9.4.1 flow cells, generating 173 Gb of reads representing 81X effective genome coverage (12,173,706 total reads, 173,867,637,954 bp total yield, 14,282 bp mean read length, 10,552 bp median read length, 22,685 bp N50 read length)."

"We used the basecaller Guppy v5.0.12 for the libraries PAG52988 and PAG53246 and Guppy v5.0.13 for the library PAH65658, both in "high-accuracy mode"."

6) The "BUSCO - proteins from TOGA annotation" in Supplementary Table 2 shows high duplication rate (40-41%) compared to the "BUSCO - genome (human as reference)". Why is this the case? Some explanation would be beneficial to understand the background.

We apologize for not explaining this in the submitted version. When running BUSCO in genome mode, one expects to find most universal single copy orthologs only once in the assembly. However, we also applied BUSCO in annotation mode to transcripts (proteins) that we annotated in each assembly with TOGA. TOGA considers all 39,664 transcripts of the 19,456 coding genes that are provided in the reference (human) gene annotation. Therefore, TOGA typically annotates more than one transcript per gene, which is intended to capture exon and transcript diversity. Since we provide all annotated transcripts to BUSCO, BUSCO often detects several hits of the single copy orthologs in the annotation. In other words, when providing several transcripts per gene, only the total number of detected BUSCO genes matters.

We clarified this in the methods:

"It is worth noting that applying BUSCO to a gene annotation (annotated proteins) produced by TOGA results in the detection of many duplicated genes because comprehensive annotations frequently include more than one transcript (splice variant) per gene. Since this does not indicate a problem but rather a comprehensive transcript annotation, we only reported the number of completely detected BUSCO genes in TOGA annotations in the Supplementary Tables 3 and 5."
We updated Supplementary Tables 3 and 5 to only list a single column "Complete".

7) The Github repo for sharing codes has no information on the genome assembly, scaffolding, curation, polishing and validation. The community will benefit from sharing exact parameters used for establishing and assessing the assemblies.

We have now added all the commands and the exact parameters used during the assembly process to our GitHub repository for this manuscript https://github.com/ariadnamorales/2023_Bat1Kimmunity#--genome-assembly. This includes all parameters for contig assembly (HiFiasm assemblies, HiCanu assemblies, Canu ONT assemblies, Medaka ONT polishing, and to purge duplications), Scaffolding (Scaff10X, Salsa2, and commands for Manual curation), and Polishing.
All assemblies and all raw sequencing data are now also available on NCBI.

- *Aselliscus stoliczkanus*: PRJNA949177
- *Doryrhina cyclops*: PRJNA938463
- *Hipposideros larvatus*: PRJNA938461
- *Rhinolophus affinis*: PRJNA938462
- *Rhinolophus perniger lanosus*: PRJNA955779
- *Rhinolophus yonghoiseni*: PRJNA938455
- *Rhinolophus trifoliatus*: PRJNA939732
- *Rhinopoma microphyllum*: PRJNA971926
- *Megaderma spasma*: PRJNA940731
- *Mops condylurus*: PRJNA949178

Please note that we created the umbrella projects for each assembly. At the moment there are only BioProjects, BioSamples and SRA datasets on NCBI and no assemblies are available yet. The internal processing times at NCBI tend to take somewhere between 1-6 months to get the assemblies in a format that can be published and this timeframe does not depend on us.

In addition to assembly, we also provide in the GitHub repository the commands and parameters for the downstream steps, including repeatMasking, genome alignments, running TOGA, phylogenomics, selection screens and enrichment analyses.

Referee #2 comparative genomics

This is a very interesting paper describing 10 new very high-quality genome assemblies for 10 bat species, and exploring selection on immune related pathways in the bat lineage compared to other mammals. The results are novel and significant, but I have concerns about the clarity of the manuscript and the rigor of some of the immune related analyses. I think there may be too much focus on the sole hypothesis of selection on viral tolerance without consideration of other explanations. Overall, this is a paper of wide interest and deserving of publication in Nature.

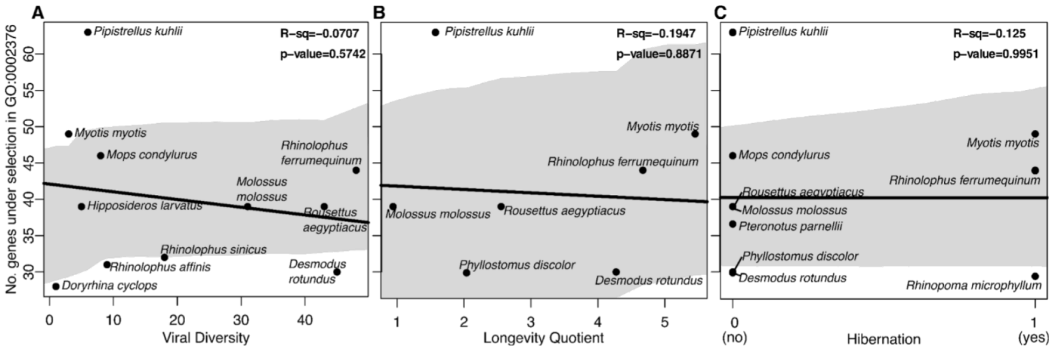
We thank the reviewer for the overall positive assessment and the helpful comments.

1) I was also disappointed that while the manuscript observes variation in selection between bat lineages, it doesn't explore that any further. For example, in figure 2C, are bat species with more immune process genes under selection also the bat species that are unusually long lived or are hibernators vs the ones with fewer immune process genes under selection? Is there any correlation with the viral diversity discovered in those lineages?

We thank the reviewer for raising these interesting questions. In the submitted manuscript, we did not take a deep exploration of lineage-specific selection patterns in bats because we focused on the coronavirus-reservoir families Rhinolophidae and Hipposideridae and the 20 bat species are not representative of chiropteran diversity. Also for many of these new species there is little information on lifespan and other relevant traits. Nevertheless, despite these limitations, we agree that it is a great idea to explore correlates of the immune gene selection patterns shown in Figure 2C.

To this end, we collected available information on viral diversity, longevity, and hibernation status, and calculated correlations between these traits and the number of immune genes selected in each species. All correlations are slightly negative but none is significant. This is likely due to the low sample size and because trait information is not available for every species in our dataset. We believe it would be interesting to re-run this analysis in the future with a substantially expanded species dataset and more phenotypic studies.

These results have been added as a new Supplementary Figure 14:



Supplementary Figure 14. Correlations between number of selected immune genes and other traits in bats.

Dispersion plots showing correlations between other relevant traits such as viral diversity, longevity or hibernation and the number of immune-related genes (GO "immune system process") under selection in the terminal species branches. Grey shading shows 95% confidence intervals.

All correlations are slightly negative but non-significant. This is potentially due to the low sample size and because trait information is not available for every species in our dataset; therefore this analysis should be repeated in the future with an expanded species dataset.

(A) Viral diversity was estimated as the number of viral families reported by the ZOVER database, identified using metagenomics or high-throughput sequencing approaches (http://www.mgc.ac.cn/DBatVir/, last accessed January 25, 2023).

(B) Longevity was measured by the longevity quotient calculated as the ratio of the observed to predicted lifespan, where predicted lifespan is based on a fitted model between longevity (measured in years) and adult body mass (measured in grams) across bats, taken from reference [8]. Data was acquired from the AnAge database (https://genomics.senescence.info/species/index.html, last accessed August 1, 2023)

(C) Hibernation was considered as 0 (no) or 1 (yes) based on published reports.

and are described in the main text:

"The number of selected immune-related genes per bat branch did not correlate with viral diversity, longevity quotient and hibernation (Supplementary Figure 14)."

Specific comments on the text:

2) The introduction includes analyses done for this paper. Specifically, the analysis of viruses detected in bats vs on mammals, and in different bat families (presented in figure 1A and 1B). If the analysis of prevalence is new, it should be moved to the results. I'm also not clear which of the comparisons is statistically significant and how that determination was made. Are the number of coronaviruses detected in bats significantly higher given that there may be a bias towards testing for viruses in bats? The text refers to # individuals, not # of species or # different populations, but presumably individuals within a species or population are not entirely independent data points. There needs to be more details on the significance of these comparisons.

We apologize if this point caused confusion. While the data underlying Figure 1A and B come from ZOVER DB, which we cite in the Figure legend, the summary analysis, the statistics (below) and the correction of the taxonomy assignment (not updated in ZOVER, our correction is described in the Methods) of this data is new. When starting this project, it was commonly assumed that bats in general and species belonging to the Rhinolophidae/Hipposideridae families in particular frequently harbor

coronaviruses; however, this was to our knowledge never systematically explored. To do such a comparison, we analyzed the ZOVER DB data. While ZOVER and the publications collected in this database rarely infer about population structure or relatedness between individuals, we explicitly only considered high-throughput metagenomics studies in our analysis to reduce ascertainment bias. We also clarified in the revised Methods that we counted the number of unique sequences of viruses belonging to coronaviruses per bat family.

The reviewer raises a valid concern that bats are much more frequently tested for harboring coronaviruses. As we now clarify in the main text, we compared how often coronaviruses were detected in comparison to other viruses in rodents and bats considering only high-throughput metagenomics studies.

As suggested, we have moved this part into the Results and quantified the differences using a two-tailed Fisher's exact test.

"**Frequency of coronaviruses in bats and rodents**

To compare how often coronaviruses were detected in comparison to other viruses in bats and rodents –another mammalian order that harbors a rich viral diversity linked to zoonotic diseases– and in species belonging to different bat families, we used data from ZOVER [4] (last access, January 25, 2023) considering only metagenomic studies. This analysis revealed that 17.8% of the viral sequences detected in bats are from coronaviruses, compared to only 1.4% for rodents (two-tailed Fisher's exact test: P=5e-50, Fig. 1A, Supplementary Table 1)."

The difference between bats and mice is highly significant (P=5e-50). The differences between Rhinolophidae / Hipposideridae and other families are also often significant, as shown in the updated Supplementary Table 1.

A two-tailed Fisher's exact test was used to test for significant differences. Significant P-values are in bold.

				Rhinolophidae vs. other bat families		Hipposideridae vs. other bat families	
	Total virus count	Coronaviridae count	Coronaviridae percentage (relative to the total number of records per family)	P-value	Odds ratio	P-value	Odds ratio
Rhinolophidae	161	67	42%	-	-	-	-
Hipposideridae	41	13	32%	0.285	1.53	-	-
Vespertilionidae	301	84	28%	**0.004**	1.84	0.5855	1.19
Pteropodidae	294	30	10%	**2.76E-14**	6.24	**0.0006**	4.06
Miniopteridae	125	12	10%	**8.15E-10**	6.67	**0.0017**	4.32
Emballonuridae	12	1	8%	**0.029**	7.77	0.1477	4.99
Molossidae	75	2	3%	**1.57E-11**	25.77	**1.88E-05**	16.50
Phyllostomidae	70	1	1%	**4.63E-12**	48.74	**6.04E-06**	31.08
Nycteridae	1	0	0%				
Rhinopomatidae	8	0	0%				
All Rodents	1261	18	1%	**4.93E-50**	0.066	Rodents vs. Bats	
All Bats	1179	211	18%				

3) The text assumes more familiarity with bat taxonomy and ecology than most readers will have. It would be more interesting with more information on the animals. For example, the authors write that they included four rhinolophid species and three hipposiderid species, but don't describe what these names refer to, or why sequencing species from both would be an important thing to do. Based on the latin names, one is a genus and one is not. There is no information on the overall number of bat genera or species or whether

the species selection represents a broad selection across the order or a specific focus on particular lineages, or exactly why these 10 species were selected.

We agree with this comment and revise the text as follows:

- We provide more background on bats and the number of recognized species at the beginning of the introduction:
  "Bats (Chiroptera) are the only mammals capable of powered flight and possess remarkable adaptations such as echolocation, exceptional longevity, and distinctive features of immunity. Chiroptera is the second most species-rich mammalian order with more than 1470 recognized species [1]."
- The number of bat families is also mentioned:
  "Coronaviruses are especially widely distributed in bats and have been detected in species from 15 of the 21 recognized bat families [4,12]."
- We introduce the common name of each bat family and each bat species mentioned in the text: "Asian horseshoe bats (family Rhinolophidae)", "Egyptian tomb bat (family Emballonuridae)", "Hipposideridae (roundleaf bats)"
- We moved the paragraph describing which species were selected for sequencing into the Results and made our rationale clear of why these species or representatives of these families were chosen. By moving this paragraph in the Results, now right after showing that Rhinolophidae and Hipposideridae harbor coronaviruses more frequently than other families (Fig. 1B), also connects these two paragraphs much better:
  "To elucidate the genomic basis of bat resistance to viral diseases, we generated reference-quality genomes for (i) bat species belonging to the families Rhinolophidae and Hipposideridae, (ii) bat species representing their sister families Rhinopomatidae and Megadermatidae, or (iii) bat species that are implicated as viral reservoirs. Specifically, we sequenced four rhinolophid (Yong Hoi Sen's woolly horseshoe bat, Rhinolophus yonghoiseni; northern woolly horseshoe bat, R. perniger lanosus; intermediate horseshoe bat, R. affinis; trefoil horseshoe bat, R. trifoliatus) and three hipposiderid species (intermediate roundleaf bat, Hipposideros larvatus; Stoliczka's trident bat, Aselliscus stoliczkanus; cyclops roundleaf bat, Doryrhina cyclops), which represent two divergent lineages within these bat families and species that mostly live in Southeast Asia, a suggested hotspot for zoonotic diseases [40]. The greater mouse-tailed bat (Rhinopoma microphyllum) in the family Rhinopomatidae and the lesser false vampire bat (Megaderma spasma) in the family Megadermatidae were sequenced to obtain high-quality genomes representing close sister families of the Rhinolophidae and Hipposideridae clade. Finally, we sequenced the Angolan free-tailed bat (Mops condylurus) (family Molossidae), which is implicated as a natural reservoir for Bombali virus (genus Orthoebolavirus) [41]."

4) I have no concerns about the genome sequencing and assembly. The genomes are exceptional. I am intrigued by the comment that most of the samples came from museum samples, and I think that deserves more focus in the main text. It has major implications for the field of genomics. What type of museum samples? What was the DNA quality? Was it of similar quality / molecular weight as from fresh samples, and did it vary depending on the type of museum sample? Were the genomes made from museum samples comparable to the other genomes? Does this show that museum samples are going to be broadly usable for producing high quality genomes?

We agree that more details regarding our samples and the DNA extraction should be provided. Therefore, we extended Supplementary Table 2 and added information on preservation, collection dates and gDNA sizes.

HMW DNA extraction	PacBio HiFi		Oxford Nanopore Technologies (ONT)		10x Genomics linked reads		Arima HiC *	Arima HiC+ **
gDNA size (Pippinpulse)	tissue	extraction	tissue	extraction	tissue	extraction	tissue	tissue
10 - > 580 kb	soft organ mix	circulomics	-	-	-	-	-	soft organ mix
50 - > 300 kb	liver	circulomics	-	-	-	-	liver	-
50 - > 500 kb	soft organ mix	circulomics	-	-	-	-	soft organ mix	-
10 - > 580 kb	liver	circulomics	-	-	-	-	liver	-
50 - > 200 kb	liver	circulomics	-	-	-	-	liver	-
10 - > 300 kb	soft organ mix	circulomics	-	-	-	-	soft organ mix	-
10 - > 150 kb	liver	circulomics	-	-	-	-	heart	-
10 - > 580 kb	liver	circulomics	-	-	-	-	-	liver
10 - > 580 kb	-	-	liver	circulomics	liver	circulomics	-	liver
10 - > 300 kb	liver	circulomics	-	-	-	-	-	liver

The collection dates for the museum samples ranged from 1992 (*Doryrhina cyclops*) to 2007 (*Rhinolophus affinis*). The samples from the other two bats (*Rhinopoma microphyllum* and *Mops condylurus*) were collected in 2013 and 2016. As mentioned in the methods

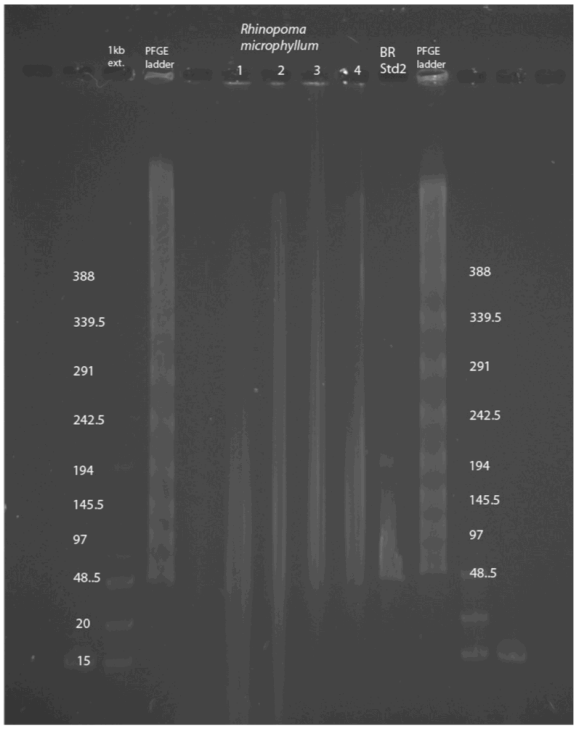
“Samples were flash frozen in liquid nitrogen after being collected and stored at -80C until further processing.”

We also added more information about the samples to the main text:

“For eight of the 10 bat species, we used tissue samples from museum collections that were kept frozen since their collection between 1992 and 2007 (Supplementary Table 2), highlighting the importance of frozen collections for biodiversity genomics [43].”

Despite the age differences of the samples, the DNA extracted from these samples passed the required quality controls and enough high molecular weight DNA was obtained for HiFi sequencing and HiC. Our study shows that even samples that are >20 years old can be used for long-read HiFi sequencing if they are adequately preserved (fresh frozen in liquid nitrogen). We added an example of a modified pulsed-field gel electrophoresis (PFGE) of the high molecular DNA extraction of *Rhinopoma microphyllum* (collected on 13.01.2013) as a new Supplementary Figure 42.

As already described in the manuscript, the only sample that yielded suboptimal results to generate a HiFi-based assembly was *Megaderma spasma*, collected on 05.05.1998. However, we cannot attribute this to sample age as the older sample from *Doryrhina cyclops* (collected 18.02.1992) produced a good HiFi yield that resulted in a high-quality chromosome-level reference genome with a contig N50 = 16.64 Mb, QV= 62, 17 + X scaffolds that can be considered chromosomes, and 98.8% of the assembly present in chromosome-level scaffolds.

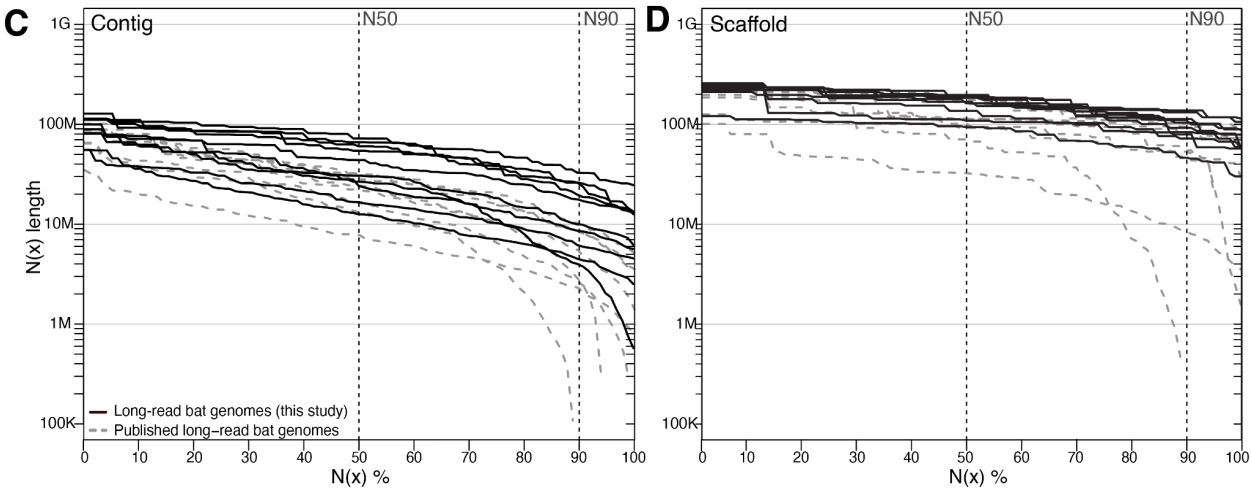


Supplementary Figure 42. High molecular weight DNA extraction.
Example of a modified pulsed-field gel electrophoresis (PFGE) showing high molecular weight DNA extracted from a *Rhinopoma microphyllum* sample that was collected in January 2013. The *Rhinopoma* gDNA is found in lane 2, HMW marker lanes are indicated, fragment sizes are in Kb.

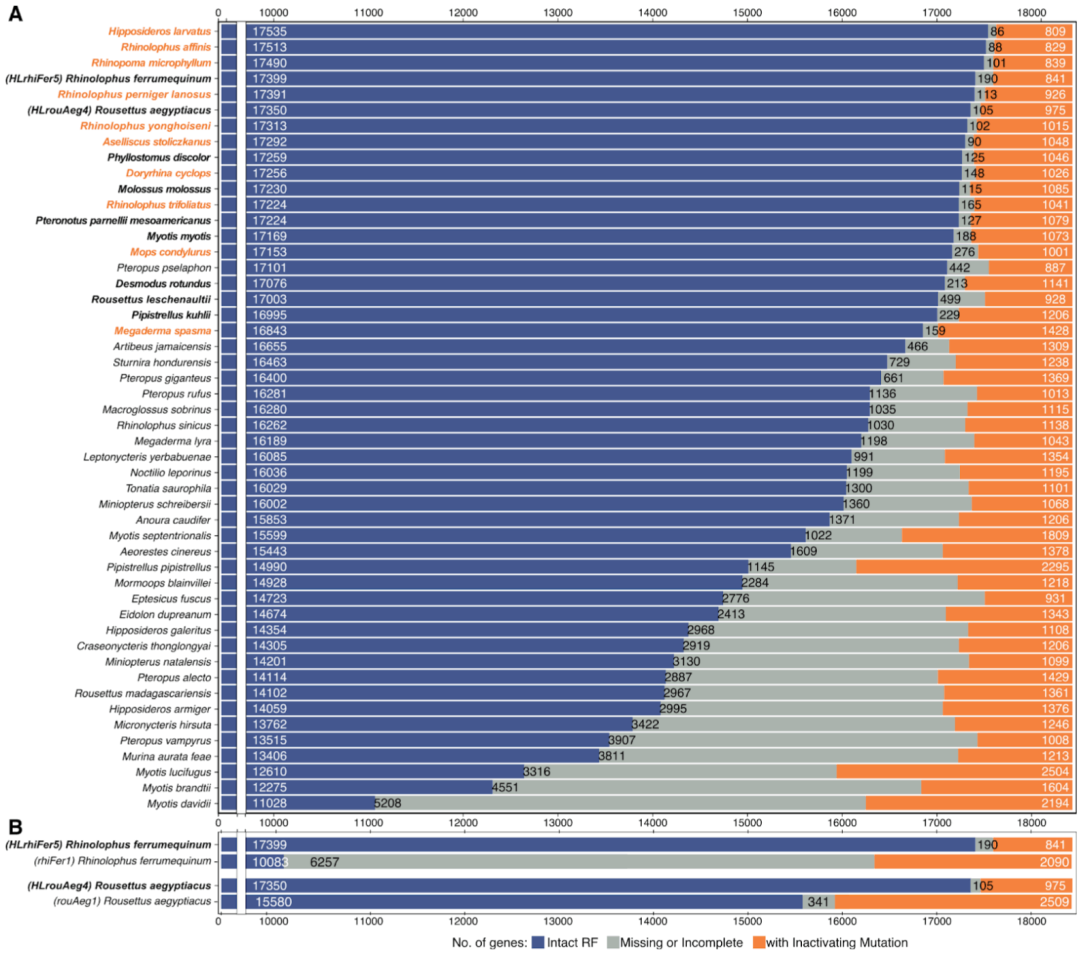
5) Figure 1C & 1D needs to be revised to be interpretable by someone with poor color vision - the colors are not easily distinguishable. In Figure 1E, I'm not sure what the interpretation should be of genes with "inactivating" mutations. Are the authors implying that these genes are not functional/lost in many bat species, or that this is most likely a reflection of genome assembly quality? It would be good to clarify that, and whether the high numbers of inactivated genes are surprising.

We apologize for the lack of visual and text clarity. It is hard to distinctly show all the lines in Figure 1C and 1D, especially in 1D where most of them overlap. Since the N(x%) lines in Figure 1C are more separated, we colored and named them, although we believe it is not possible to get 10 distinct colors that are distinguishable by colorblind people. To alleviate this, we ordered the legend in 1C according to the N50 value, as we now clarified in the legend:
"New bat assemblies are shown as solid colored lines, and they are sorted by contig N50 in the inset legend."
This means, the top line crossing the N50 mark is *Aselliscus stoliczkanus*, etc.

The main message of Figure 1C and 1D is that our new assemblies have a contiguity that is similar and often higher than the best bat assemblies available so far. As an alternative, if the reviewer is not satisfied with leaving the colors and ordering the legend according to N50, we can also present the main figure message by just using black solid lines for our new assemblies and grey dashed lines for previous assemblies. This would also convey the main message but provides less detailed information. It would look as follows and we are happy to follow the reviewer's advice:



With respect to inactivating mutations: TOGA detects and considers premature stop codons, frameshifts, splice site disruptions, and deletions of exons or entire genes as gene-inactivating mutations. The presence of such mutations either indicates that the respective gene may no longer encode a functional protein or that the gene structure has substantially changed between the reference (human) and query species (bats). However, given a clade such as bats, we can compare the number of ancestral genes having inactivating mutations or missing sequences. A clear excess in either category is then less likely to be a biological difference, and rather indicates assembly quality issues. For example, in Supplementary Figure 4, the assembly of *Pipistrellus pipistrellus* has twice as many genes with inactivating mutations (2295 vs. ~900-1100 for the HiFi assemblies), which indicates a lower base accuracy. Supplementary Figure 9 shows that assemblies of other mammal orders have similar numbers of genes with inactivating mutations, with the exception of non-human primates that are more closely-related to human (used as the reference).



Supplementary Figure 4. Status of 18,430 ancestral placental mammalian genes in bat genomes.

(A) 50 bat genomes used for phylogenetic inference.

(B) Bat genomes that we used to explore the effect of genome quality on selection screens.

We defined inactivating mutations and clarified the interpretation in the Fig. 1E legend:

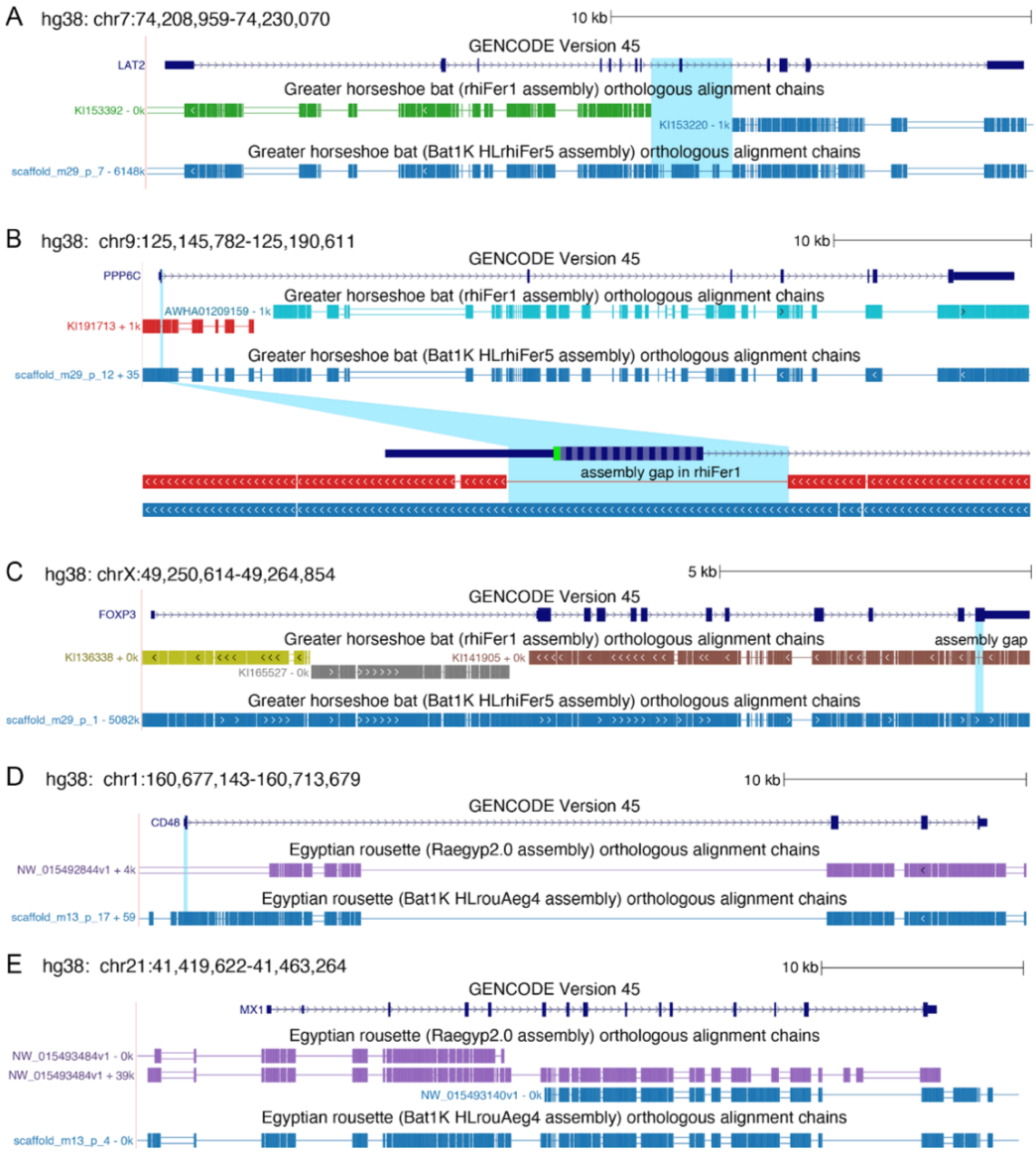
"Genes are classified by TOGA into those with an intact reading frame (blue), with gene-inactivating mutations (premature stop codons, frameshifts, splice site disruptions, and deletions of exons or entire genes; shown in orange), or missing or incomplete coding sequences due to assembly gaps or fragmentation (grey). An excess of genes with missing sequences indicates a lower assembly completeness and an excess of genes with inactivating mutations indicates a lower base accuracy."

6) After the description of the new genomes, however, the manuscript gets a bit confusing. The title of the paper implies that the authors were able to detect adaptations for immune tolerance because the new genomes are "reference quality". In the manuscript, there doesn't seem to be a clear justification of this. To detect selection, the authors used genomes for 20 bat genomes and 95 species from other mammalian orders, but it is not clear whether the fact that the new bat genomes are high quality mattered for the results. If the analysis had been done with lower quality genomes, would the findings have been any different?

We thank the reviewer for raising this point. Previous studies have shown that short read based assemblies contain many false gene duplications and losses (Kim et al. 2022; Ko et al. 2022), hampering comparative analysis. We agree that further evaluating the effect of assembly quality on the results of genome-wide selection screens is important.
As suggested, we re-ran our analysis after replacing high-quality assemblies with previously-published lower quality assemblies of the same bat species (same species but different assembly tests exclude other confounding factors). While almost all species included in our screen have only a single genome assembly, for two bat species (*Rhinolophus ferrumequinum* and *Rousettus aegyptiacus*) an assembly with a lower quality was available.
We repeat-modeled and repeat-masked these two assemblies, aligned them to human, and ran TOGA. The new panel B in Supplementary Figure 4 (screenshot in point 5 above) shows that these two assemblies have fewer genes with an intact reading frame. In particular, the previous *R. ferrumequinum* has many genes with missing sequences, and the previous *R. aegyptiacus* assembly has an excess of inactivating mutations, indicative of base and assembly errors.

We then re-ran the screen for positive selection, changing one species at a time, keeping the other 114 assemblies the same. This identified 272 vs. 133 genes under positive selection for the high vs. lower quality assembly of *R. ferrumequinum*, and 299 vs. 194 genes under positive selection for the high vs. lower quality assembly of *R. aegyptiacus*. Thus, the number of genes under selection in the *R. ferrumequinum* and the *R. aegyptiacus* branch decreased substantially, showing that lower assembly quality hampers the identification of selected genes.

We added a new Supplementary Figure 6, where we illustrate the range of problems (missing sequences, assembly errors, false duplications) with examples of five immune-related genes where the positive selection signal would have been missed with a lower-quality assembly.



Supplementary Figure 6. Lower assembly quality leads to missed signals of gene selection.

To explore the effect of assembly quality on results from genome-wide selection screens, we replaced the high-quality Bat1K assembly (HLrhiFer5) of the Greater horseshoe bat (*Rhinolophus ferrumequinum*) [1] with a previous short read based assembly (rhiFer1) of the same species [2] that has a higher degree of incompleteness and fragmentation (panel A-C). We

These tests show that lower quality assemblies make it harder to comprehensively identify positive selection signals in a genome-wide screen. We now also cite the VGP paper that demonstrated the importance of high-quality genomes for correct gene annotations as a prerequisite for selection screens. We added this to the main text by writing:

“High assembly completeness and quality thus facilitates comprehensive screens for gene-wise positive selection (Supplementary Figure 6) and is also a prerequisite for a comprehensive annotation of transposable elements [46–48].”

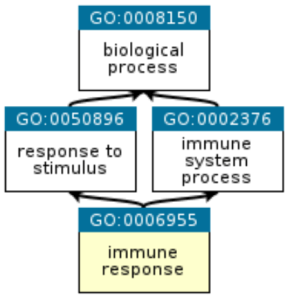
7) I appreciated the section titled "Selection in immune genes is most prevalent in bats" which assessed whether the enrichment for selection at immune-related genes in bats was exceptional compared to other mammalian orders. However, the lack of numbers was concerning. The main text should include some indication of which results are significant after correction and p-values, but this is missing from both the text and figure 3. For the heatmap in figure 2 A, D and E, I'm not sure which results / boxes are significant, and whether NA means that no genes overlapped or the enrichment was not significant.
The bats really don't look all that different from the rodents in figure 2A. How meaningful is it that bats have the "strongest enrichment" or is it more that bats and rodents have the strongest enrichment and then there is everyone else?

We apologize for the lack of clarity in these figures and the text, and we improved them as follows:

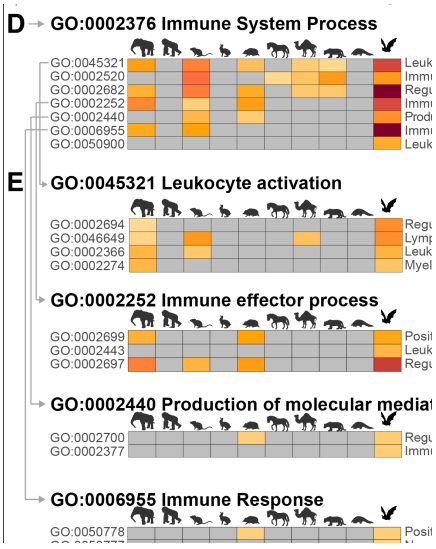
- The enrichment analysis with gProfiler corrects for multiple testing, and we now clarified that in the Figures and the text.
- In Figure 2, we have removed NA, which meant that there was not a single gene under selection for a particular GO term. Instead, we now use grey color for every GO term that is not significantly enriched at P=0.05 after multiple test correction, which by definition includes the cases with 0 selected genes. That means, every cell with color stands for a significant enrichment. We also adjusted Supplementary Figure 10-12 and 21 accordingly. The legend of Figure 2 has been adjusted:
  "(A) Functional enrichments of genes under selection in mammalian orders (columns). All high-level gene ontology (GO) terms representing different biological processes were tested (rows), but only terms significantly enriched in at least one mammalian order are shown. Cells with color indicate a significant enrichment after correcting for multiple testing."
- We added the corrected P-values to the main text at several places.
  "we found that bats have the most significant enrichment for "immune system process" (GO:0002376, corrected P=2.02E-16), followed by rodents (an order also known to harbor diverse viruses, corrected P=6.75E-11; Fig. 2A)."
  As pointed out by the reviewer, listing these corrected P-values makes it clearer than from the figure alone that bats have much more significant enrichment of selection in immune genes, compared to rodents, afrotheria and all other clades.
  We also added the P-values to the main text describing the Figure 2D results: "This showed that, compared to other mammalian orders, genes under selection in Chiroptera have the most significant enrichments for several child terms, including "immune response" (GO:0006955, corrected P=1.44E-10), "regulation of immune system process" (GO:0002682, corrected P=2.12E-11), "immune effector process" (GO:0002252, corrected P=5.66E-08) and "leukocyte activation" (GO:0045321, corrected P=9.31E-09)."
- Figure 3 shows both genes under selection in bats and other genes that are important to understand the biological context. To make it clear which of these genes are under selection in bats, we revised the legend to:
  "Genes that evolved under positive selection in the ancestral Chiroptera branch (white font), the stem branch of Rhinolophidae and Hipposideridae (yellow font), or the Rhinolophidae stem branch (orange font) are shown with a dark blue background."

8) While the manuscript focuses on immune genes, the enrichment for Response to stimulus and other processes seems stronger. Are these enrichments also driven by immune related genes? It would be helpful to have the equivalent of figure 2C and 2D for other biological processes with significant enrichments and information on whether the selection for those processes is similarly timed.
Figure S8 is supposed to show all child terms of high-level GO terms in Figure 2. All of them appear to be immune system related, though - how were the terms chosen for inclusion in this figure?

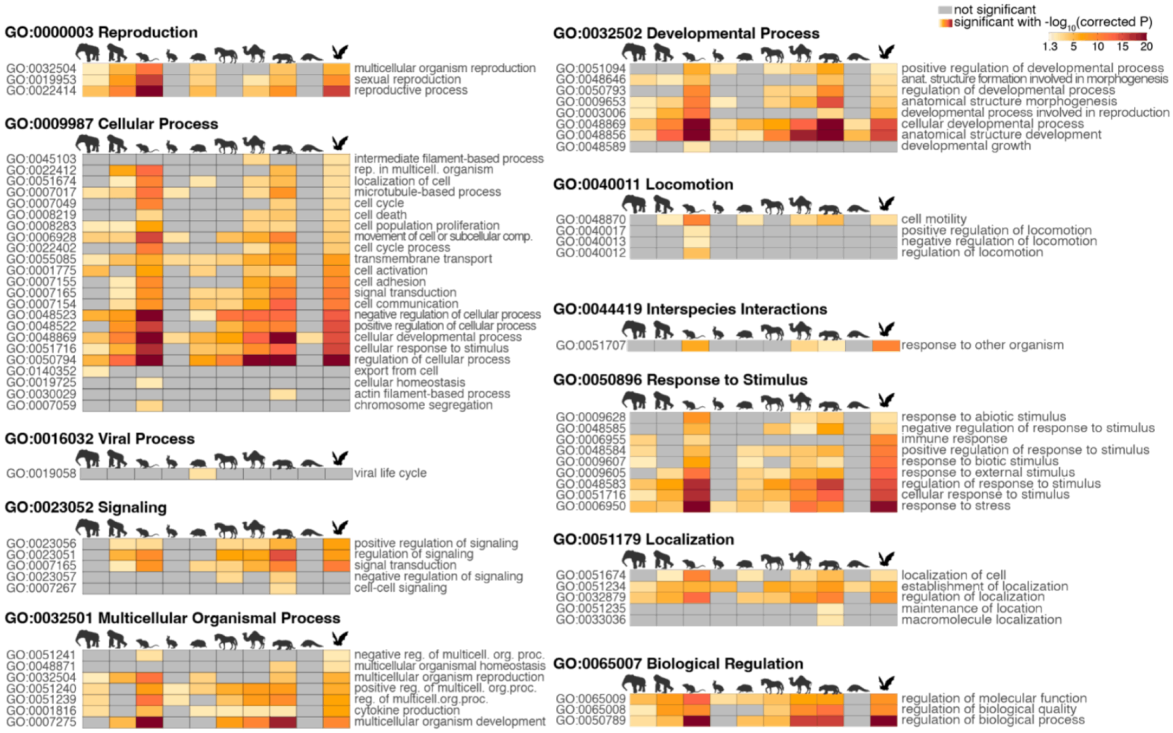
The reviewer is correct that for bats the top-level GO term "response to stimulus" is more significant than the top-level GO term "immune system process" (corrected P=1.04E-24 vs. 2.02E-16). Indeed, the enrichment of both top-level terms is best explained by their common child term "immune response", which has by far the strongest enrichment in bats (corrected P=1.44E-10), followed by afrotherians and rodents that both have much higher corrected P values (P=0.0004 and P=0.0009), as shown in Figure 2D. In contrast to bats, rodents have much stronger enrichments for non-immune related child terms of "response to stimulus", which include "response to abiotic stimulus" (corrected P=2.40E-09 for rodents vs. corrected P=0.006 for bats), "response to radiation" (only rodents with corrected P=5.63E-07), "response to light stimulus" (only rodents with corrected P=0.0012) and "response to wounding" (only rodents with corrected P=0.007).



We added to the main text:
"The Chiroptera enrichment for "immune response" also contributes to the enrichment of the high-level GO process "response to stimulus" (GO:0050896, corrected P=1.04E-24; Fig. 2A)."
To make it clearer that Figure 2D and E show GO child terms of terms presented in 2A, we added connecting lines between the panels and terms, as shown here:



We apologize for a mistake in generating Supplementary Figure 8 (which is now 10). As correctly noticed by the reviewer, this figure showed only child terms of “immune system process”, which are already shown in Figure 2D. To correct this, we replaced this figure with a new one that shows the enrichment results for all child terms of the other 11 top-level GO terms.



Supplementary Figure 10. Enrichments for genes under selection and child terms high-level GO Biological Process terms.
Heatmaps show enrichment results for the child terms of the 11 high-level GO terms that are shown in Figure 2A (child terms of “immune system process” are already shown in Figure 2D). Enrichment tests for genes under selection in the different mammalian groups (columns) were performed in gProfiler. Only child terms that have a significant enrichment in at least one mammalian order are shown. Cells with color indicate a significant enrichment after correcting for multiple testing. Supplementary Table 7 provides the full gProfiler output, including all significant p-values for all groups.

9) The authors state “Together, this suggests that immune system changes originated early in the chiropteran lineage and coincided with the evolution of powered flight.” This implies causality which is not supported by data, and it needs more context - flight hasn’t been mentioned since the introduction. Are there other traits that evolved at the same time that would also be explanations?

We did not intend to imply that flight and immune system changes are causally connected. Since we do not know when flight evolved in the ancestral Chiroptera lineage, we rephrased the sentence to: “Together, this suggests that immune system changes are distinctive adaptations that originated early in the chiropteran lineage on the branch where powered flight also evolved”.

To the best of our knowledge, there are no other prominent traits that were present in the chiropteran ancestor, potentially because this ancestral lineage is also less well characterized due to a sparsity of representative fossils. While there is contrasting evidence whether echolocation was an ancestral trait that got later lost in Pteropodidae, it is undebated that powered flight, as a trait shared by all bats, evolved once in this ancestral lineage.

10) The section titled "Relevant immune-related changes in bats" is very problematic and needs extensive revision. Right now, it feels like the authors are cherry picking their way through a series of vignettes chosen to fit their overarching point that immune genes are under selection (they write "we intersected knowledge about genes involved in immune responses to corona- and other viruses with positively selected genes"). If that is all this is, it should be relegated to the discussion. It's not clear how they chose which genes to discuss, or whether there is more selection on coronavirus response genes in bats than in other orders. I can't figure out how many strongly selected genes they are not discussing because they can't make an immune related story about them. If their argument is that specific immune response pathways are under selection, there should be some kind of analysis showing that that pathway has an excessive number of selected genes. It's also not always clear what information is based on previous studies and what statements are related to observations of the data in this paper. I think most of the text is discussing previous work, and not the results from this paper, which should be the focus.

We apologize for not making it clear that this Results section is tightly connected to the above-described GO term analysis and it serves two purposes. First, while our GO analysis enabled a comprehensive and unbiased comparison, it is also a rather high level analysis. In particular, Figure 2D and E do not present more specific and informative GO terms (all listed in Supplementary Table 7) and the underlying genes as new experimental targets are not mentioned. Therefore, we thought the paper would come short if we do not analyze more informative enrichments that are unique or strongest for bats, together with key genes that underlie these enrichments. Second, GO does not capture every gene that has an immune-related function, in particular for genes where recent COVID19 studies revealed an immunity role for the first time. As an example, the RIG-I regulating *LRRC25* and the NF-κB signaling regulator *TNFAIP2* genes are not assigned any immune- or virus-related GO terms. To correct this shortcoming, we

- integrated information from pathway databases such as WikiPathways (providing a COVID19-network) and KEGG,
- screened the literature for information not present in databases, and
- had the virologists on our authors list shortlist those key genes for which their role in the immune system is reasonably well understood.

Together, while we cannot mention every selected gene, the selection patterns we reveal have never been discovered before and the selected genes are promising targets for additional experimental studies.

To address the reviewer's comment, we

1. reworked the rationale of this section, explicitly describing the goals and additional analyses: "To gain insights into immuno-modulatory pathways that could contribute to bat tolerance against corona- and other viruses, we explored more specific enrichments from Gene Ontology and other pathway databases, including the SARS-CoV-2 signaling pathway map (WP5115) [56]. Since recent studies have revealed new genes with important immunity- and inflammation-related roles, we also integrated knowledge from the literature. Focussing on genes under selection in the ancestral

chiropteran branch (abbreviated as C), the common stem branch of Rhinolophidae and Hipposideridae (RH), and the Rhinolophidae stem branch (R) revealed promising experimental targets linked to …"

2. made it clear that the processes and genes we present resulted from our analysis of specific enrichments that (i) are either uniquely observed for genes selected in bats or (ii) have the most significant enrichment for bats. This also shows that, for these processes, bats have an excessive number of genes under selection (more than expected by chance).

   "Interestingly, our analysis showed that enrichment of "innate immune response" (GO:0045087, corrected P=1.37E-05) is only observed for genes under selection in the set of 20 bats (Fig. 2E), and "inflammatory response" (GO:0006954, corrected P=2.51E-14) and "regulation of cytokine production" (GO:0001817, corrected P=1.58E-07) have the most significant enrichments for bats (Supplementary Table 7). These GO terms include several genes that are involved in detecting pathogen-associated molecular patterns and regulating inflammatory immune responses (Fig. 3B), and that evolved under selection in bats. Among them …,

   "Inflammation triggers the cellular release of chemokines that direct the migration of leukocytes to sites of infection and our analysis showed that "leukocyte migration" (GO:0050900, corrected P=0.0016) is only enriched for genes selected in bats compared with other mammals."

   "A key effector process of the activated immune system is the complement system and "Regulation of immune effector process" (GO:0002697, corrected P=1.55E-08; Fig. 2E), "Complement system" (WikiPathways WP2806, corrected P=8.92E-08) and "Complement and coagulation cascades" (KEGG:04610, corrected P=0.0003; Supplementary Table 7) have the most significant enrichment for genes selected in bats."

11) The section on "ISG15 of rhinolophid and hipposiderid bats vary in antiviral activity" is really intriguing, and their results are a beautiful demonstration of how complicated it is to connect a particular change to a particular phenotype (even an amino acid change) when you are working in an evolved and complex system. I think it would be valuable if that aspect of the results were given more prominence. The major missing component, though, is a justification for why this particular mutation in this particular gene was chosen for functional investigation, given the many different immune genes described in the previous section. It would be helpful to get more information on that.

There were several reasons why ISG15 appeared to us as a very promising experimental target. First, in Oct 2021, it was shown that ISG15 acts as a proinflammatory factor implicated in the cytokine storm that can lead to death in patients developing severe COVID-19. This proinflammatory role of ISG15 depends on homodimer formation, for which the Cys78 residue is required. Second, by inspecting our codon alignments, we discovered that precisely this Cys is deleted in the two bat families (Rhinolophidae and Hipposideridae) where coronaviruses are most frequently detected (Fig. 1B). Third, ISG15 is a reasonably short gene (165 amino acid residues in human), which made our systematic experiments feasible that now test an extended set of 17 ISG15 constructs of a total of 13 species. Together, while ISG15 is a very well studied protein, our previous and new experiments revealed new insights into functional differences of ISG15 in humans and bats.

We realized that the previous paragraph introducing ISG15 was convoluted and did not make our motivation clear. Therefore, we have rewritten this part, put it under its own section heading “ISG15 of rhinolophid and hipposiderid bats lacks a conserved Cys residue”,
and gave this section a clear structure that first introduces the roles ISG15 has inside (ISGylation) and outside (pro-inflammatory cytokine function) the cell, and then describes the importance of ISG15 in monomeric and dimeric form. We also highlight the importance of Cys78 for ISG15’s structure and function relationship, which motivates our experiments:
“As mutating this Cys78 will prevent ISG15’ homodimer accumulation, altering extracellular cytokine activity and possibly enhancing ISGylation, (dimerized ISG15 cannot be used for ISGylation) [116,117], we hypothesized that the Cys78 deletion may alter the function of rhinolophid and hipposiderid ISG15.”

12) The ISG15 section also was not very readable and had too many acronyms. It might help if each paragraph started with a sentence giving the big finding / main point before delving into the methods.

We thank the reviewer for pointing this out and we improved readability in the experimental ISG15 section as follows:

1. We restructured the paragraphs, also to incorporate the new experiments. After a section describing the discovery of the Cys78 deletion, we now have a separate section showing computationally and experimentally that the absence of Cys78 in bat ISG15 prevents stable dimer formation. The following experimental sections are now separated by the virus (VSV, influenza, coronaviruses) that we tested. After VSV, we outline the viruses that were tested
“We next examined how human, R. affinis (with and without Cys78) and M. condylurus ISG15 affects viral production of three other viruses: Influenza A virus (IAV), human coronavirus 229E (HCoV-229E), and SARS-CoV-2. “
and the following paragraphs that describe the results for one virus start with signal words “First”, “Second” etc
2. We split and simplified rather complex sentences in these paragraphs.
3. To avoid wordiness, commonly-used abbreviations are needed. However, we explain each of them and provide context:
    - … HEK293 (human immortalized embryonic kidney) cells
    - … Vesicular Stomatitis virus (VSV), a representative of the Rhabdoviridae family that is common amongst bats
    - … three other viruses: Influenza A virus (IAV), human coronavirus 229E (HCoV-229E), and SARS-CoV-2. First, we tested IAV as a member of the Orthomyxoviridae family that is commonly identified in bats.

Since we use official gene symbols (https://www.genenames.org/), we decided to not provide the full gene names for each gene mentioned in the text. However, Supplementary Table 6 lists the gene names (column R) together with gene ontology, pathway, expression and disease annotations (columns S to AG).

Discussion

13) The statement “However, compared to other mammalian orders, bats exhibit the strongest enrichments for immune gene selection, providing genomic evidence that bats possess unique immune system

adaptations." is too strong. An enrichment for selection at immune genes alone is not evidence of unique adaptations.

We agree with the reviewer and have replaced 'unique immune system adaptations' with 'pronounced immune system adaptations', which reflects our finding that immune gene selection is most prevalent in bats.

14) "This supports that the evolution of powered flight and immune system adaptations are linked" - is powered flight the only thing different about Chiroptera? Is it really evidence for anything more that cooccurence? More support/context for this statement would be helpful.

Powered flight is a key innovation that evolved in the ancestral bat lineage. Flight may be linked to immune system changes, because "powered flight requires high metabolic rates and many by-products of rapid metabolism and cellular stress can activate the immune system, greater viral tolerance could have evolved as a byproduct of immune adaptations to counter flight-induced sterile inflammation [24,34]", as mentioned in the introduction. If there is a connection between flight and immune system changes, one would expect to find signatures of immune system adaptations in the ancestral bat branch, which is what we found in this study. We agree that this is a co-occurrence pattern, but we find it worthwhile to mention it in the Discussion.

To make the connection to the previous sentence clearer, we now rephrase this part, which now reads: "A regression model to estimate the expected number of selected genes showed that the ancestral chiropteran branch exhibits more immune gene selection than expected. Since powered flight also evolved in the ancestral chiropteran lineage, this supports a connection between flight and immune system adaptations [24,132,133]; however, it remains to be determined whether flight is directly or indirectly linked to immune system changes."

15) "While we applied the regression model specifically to immune genes, this approach could be a generally-applicable strategy to reveal lineages (branches) having an excess of selection on genes belonging to particular functional categories." I think (unless the regression model is very arduous to apply) it would be reasonable for a paper of this scope to do this analysis of other functional categories as well.

This is an excellent suggestion. We have now also explored whether genes selected in bats and other mammalian orders are enriched for other gene ontologies (GOs) related to relevant traits known in bats. We extracted a list of genes that can be relevant for hibernation, echolocation, and longevity. We applied the same methods to extract gene counts per branch and per GO, tested correlation models between branch lengths and gene counts, selected the model that best fits the data, and calculated the residuals based on such a model.
Notably, we did not find the same results observed with the immune-related GOs and SARS-related pathways, where the ancestral Chiroptera branch shows more genes under selection than expected.
GOs related to hibernation and dormancy (GO:0042750, GO:0022611) did not yield enough genes, and thus, the results of correlation models were not significant.
For echolocation-related genes, we merged the genes annotated in sensory perception of sound (GO:0007605) and ear development (GO:0043583). Results do not show more observed genes under selection than expected in the ancestral Chiroptera branch.

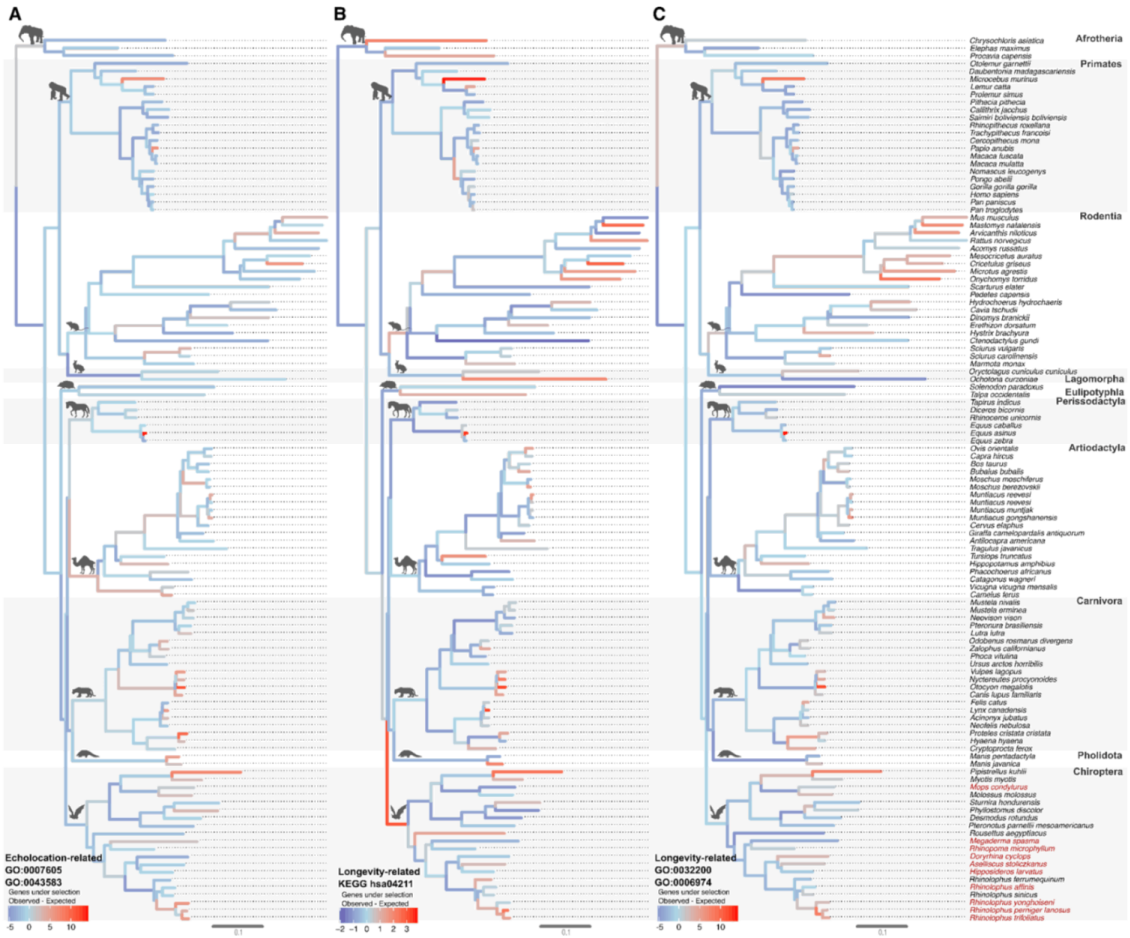
For longevity, we generated two sets of genes. First, we used those annotated in the KEGG “Longevity pathway” (hsa04211); second, we combined genes annotated in telomere organization (GO:0032200) and DNA damage response (GO:0006974). Interestingly, the ancestral Chiroptera branch shows a higher than expected number of genes under selection when we fit a model based on the KEGG “Longevity pathway.” However, this pathway includes 89 genes, therefore we also investigated whether immune-related genes drive this result. Indeed, when we remove the 30 genes that are also part of the GO:0002376 “Immune System Process”, the ancestral Chiroptera branch only shows two genes under selection.

Moreover, when we examined other GOs linked to longevity, such as telomere organization and DNA damage response, the ancestral chiropteran branch did not show an excess of observed minus expected genes under selection.

Finally, we fitted models that include all the genes under selection to rule out that genes in bats are generally more often under selection (new Supplementary Figure 17). As with the previous GOs, this test did not find a signal of more genes under selection in the ancestral chiropteran branch. These results further support our findings that since their early origins, bats have evolved more immune related adaptations than other mammals. We now mention such results in the manuscript

“In contrast, genes with functions relevant for other bat adaptations (longevity and echolocation) show no excess of selection on the ancestral chiropteran branch, although we observed a small excess of selected genes for the KEGG “Longevity regulating pathway” (Supplementary Figure 20).”

and added Supplementary Figure20. The raw values are given in Supplementary Tables 8 and 9.



Supplementary Figure 20. Per-branch signal of selection for genes with functions potentially relevant for echolocation and longevity.

Expected numbers of selected genes in the given gene set were calculated from the regression models shown in Supplementary Tables 8-9. Branches are color-coded based on the difference between the observed and expected number of selected genes. New Bat1K genomes are in red font.

(A) Genes with functions potentially relevant for echolocation, defined as genes with GO terms “sensory perception of sound” (GO:0007605) and “ear development” (GO:0043583).

(B) Genes with functions potentially relevant for longevity, taken from the KEGG “Longevity regulating pathway” (hsa04211: https://www.genome.jp/entry/pathway+hsa04211 last access November 15, 2023), which contains 89 genes. Interestingly, the ancestral Chiroptera branch shows a higher than expected number of genes under selection (4 observed vs. 1.3 expected). However, the hsa04211 pathway also includes 30 immune-related genes (genes that are also annotated with GO:0002376 “Immune system process”). Therefore, we tested if this signal is still present when removing the 30 immune-related genes. After removing these genes and fitting the regression model for the reduced gene set, we found that the ancestral Chiroptera

Referee #3 virology

Bats, known as natural reservoirs for viruses, have been found to survive many viruses that are highly lethal to humans, such as SARS-CoV, SARS-CoV-2, MERS-CoV, Marburg, and henipaviruses. Several studies have shown that bats have significant functional differences in their innate and adaptive immune systems compared to other mammals, enhancing their antiviral immune response and reducing inflammatory signaling. However, the genetic and molecular mechanisms responsible for bat resistance to viral disease remain largely unknown. Morales et al. sequenced high-quality genomes for ten bat species, including those that harbor coronaviruses, to investigate the genomic basis of disease resistance. Their analysis indicated that bats possess higher immune gene selection than other mammals, including genes linked to viral detection and entry, inflammation regulation, antiviral mechanisms. They found that rhinolophid and hipposiderid bats display a deletion of a cysteine residue critical for ISG15 homodimer formation. The authors propose that this cysteine deletion in the bat ISG15 protein results in increased intracellular protein conjugation and decreased secretion of ISG15 into the extracellular space, thereby limiting the extracellular pro-inflammatory role of ISG15.

The authors have successfully built high-quality genomes for 10 bat species using long-read and long-range sequencing technologies. Compared to low-coverage or short-read NGS technologies, their approach significantly improves the contiguity of genome assemblies and enables better resolution of repetitive genome loci, where important immune gene loci reside. The resulting assembly and annotation, along with accompanying genomes, are of exceptional quality. Furthermore, the authors conducted a genome-wide screen and analyzed orthologous genes that have undergone positive selection across various mammalian orders, providing strong genetic evidence of positive selection of immune-related genes in bats. Overall, this aspect of the study is expected to provide a highly valuable asset for investigating the evolutionary history and genetic underpinnings of bat biology and adaptations.

The major weakness of the study relates to the data on the proposed antiviral mechanism of bat ISG15, which do not convincingly support the conclusion that rhinolophid and hipposiderid bat ISG15 with a cysteine deletion remains intracellular and exhibits increased ISGylation to dampen aggressive inflammatory responses. It is unclear why ISG15 in Rhinolophidae and Hipposideridae did not show a consistent antiviral difference when compared to human ISG15, although the authors propose that this may be due to additional mutations in individual bats. Moreover, the data on the expression and conjugation of overexpressed bat ISG15 in human cells are not convincing, and there is a concern about antibody compatibility in detecting bat ISG15. These data require extensive additional controls, and also confirmation experiments in bat cells, such as whether restoring Cys78 of bat ISG15 alters its ISGylation and secretion capacity in bat cells (see also specific comments below).

> We thank the reviewer for the valuable feedback and the insightful suggestions. We have conducted many new experiments to address all points raised and believe that the revised manuscript is much stronger now.
> In particular, we have synthesized Myc-tagged constructs for ISG15 of an extended list of species to avoid cross-species antibody differences and this allows us to only capture the overexpressed Myc-tagged and not the endogenous ISG15. We repeated all previous experiments, performed new experiments, and have restructured the experimental sections in the manuscript. The experimental results are now presented in two figures (Figure 4 and 5).
> We respond to each individual point in the following.

Major points:

1.As shown in Figure 4A, some bats still retain the cysteine residue (Cys78 in human). To validate whether this cysteine is indeed essential for bat ISG15's antiviral function and its ability to limit pro-inflammatory responses, the authors should also include one representative bat ISG15 which contains Cys78 in the experiments in Figures 4B-4H.

This is an excellent suggestion. We have now synthesized ISG15 of three additional bats that are outside the rhinolophid and hipposiderid clade and naturally have Cys78: *Mops condylurus*, *Myotis myotis* and *Rousettus aegyptiacus*. To ensure consistency and minimize variations among results, we repeated all experiments with ISG15 constructs including the new bat species and representative rhinolophids and hipposiderids, repeated infections (Fig. 4D-G and Fig. 5A-C), quantifications and western blots (Fig. 4B,C,H-K). Since we had to repeat all experiments for comparability and since we saw the biggest difference in the production of viruses, we measured production of the four different tested viruses in the supernatant instead of %infected cells, for consistency.

Our results show that while the presence of Cys78 confers stable dimer formation, also in *Mops condylurus* that naturally contains a Cys at this position, the presence of the Cys78 does not directly correlate to secretion from cells nor antiviral activity. For example, *M. myotis* ISG15 is less antiviral against IAV compared with ISG15 of *M. condylurus* and *R. aegyptiacus* (Fig. 5A). Furthermore, we noted key differences in the behavior of two human mutants that replace (C78A) or delete Cys78 (requested in point 3 below), indicating the structure around this residue may well be important, in addition to stable dimerization formation. Additionally we show that in our cellular system, the induction of cytokines or enhancement of proinflammatory signaling does not play a role, and the presence of the Cys78 does not significantly impact innate immune induction (Supplementary Figures 36 and 37).

Our finding that ISG15 of *M. condylurus* and *R. aegyptiacus* (both with a natural Cys78) is potent against IAVs or VSV, which is closely related to RABV and a virus found in *M. condylurus*, indicate matched evolutionary trajectories for ISG15-mediated function towards viruses that are more commonly associated with these bat families. This is supported by the lack of a significant antiviral activity of the Cys78-containing ISG15 of these three newly-tested bats against SARS-CoV-2 (Fig. 5C), as betacoronaviruses are rarely found in these bat families. Together, these new experiments show that there are species- and virus-specific differences not explained by the presence or absence of Cys78, indicating that additional mutations in individual bats (Fig. 4A) also affect ISG15's antiviral activity.

2.Figure 4E. It is somewhat puzzling that, despite having a Cys78 deletion like most other bat ISG15s, the ISG15 of Aselliscus stoliczkanus and Rhinolophus trifoliatus do not show an increased antiviral activity compared to human ISG15 against SARS-CoV-2. However, Rhinolophus yonghoiseni, which is closely related to Rhinolophus trifoliatus, exhibits a significantly enhanced antiviral activity. Does this mean that the mutations between these two bat ISG15 proteins account for their antiviral function against SARS-CoV-2? It is suggested to look at the specific sequence differences and conduct mutagenesis to explain this phenomenon.

The reviewer raises an interesting point. As mentioned above, our data shows that species-specific mutations beyond the Cys78 deletion contribute to the variable antiviral activity against SARS-CoV-2 and other viruses. To address this comment, we aligned the ISG15 protein of these closely-related

bats, which shows that there are 11 amino acid differences (white or grey background in the alignment figure), consistent with ISG15 evolving rather fast, even between closely-related species.

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R.trifoliatus       MIGELKVKMLSGKDfsVPLrDSMLTAELKQRVAQETgVPAFQQRLISQSSGeELQDRATL
R.yonghoiseni       MIGELKVKMLSGKNllVPLtDSMLTAELKQRIAQETsVPAFQQRLISQSSGaELQDRATL

R.trifoliatus       VGQGLRPGSTVLLVVLSDDPLSILVKNEKGRTRVYEVRRTQTVDELKQQVhrQEGTPADQ
R.yonghoiseni       VGQGLRPGSTVLLVVLSDDPLSILVKNEKGRTRVYEVRRTQTVDELKQQVqgQEGTPADQ

R.trifoliatus       FWLSFQGKPMEDQYQLGKYDLRPHCTVLMNLRLRGGGAGlEGSR
R.yonghoiseni       FWLSFQGKPMEDQHQLGKYDLRPYCTVLMNLRLRGGGAGpEGSR
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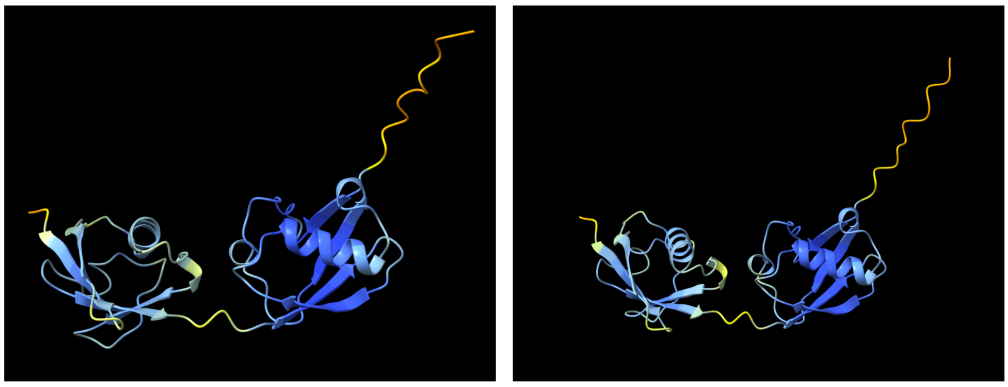
We tried to narrow the number of candidates down by searching for structurally-relevant amino acid differences. However, Alphafold shows no visible differences in the top seed model between the ISG15 protein structural elements in these species, particularly the C-Lobe (right side) that binds the cleavage site (see the figure below), making all 11 amino acids as well as certain combinations thereof potential candidates to explain the differences in SARS-CoV-2 antiviral activity. This results in a very large number of constructs (11 for single amino acids, 55 for amino acid pairs) that are not not feasible to test in a BSL3 lab, which is required for SARS-CoV-2 experiments. We therefore think that exploring which set of amino acid differences is causing the observed differences in antiviral activity should be the focus on a future project.

We offer two additional pieces of evidence, however, that may provide some insight. First, as requested in point 7 below, we mutated the LRGG conjugation motif to LRAA to abolish ISGylation (Fig. 5D). These mutagenesis studies show that indeed conjugation is essential for full antiviral activity against SARS-CoV-2. Second, structural modeling of *R. trifoliatus* ISG15 when bound to PLpro of SARS-CoV-2 indicates a structure most similar to human ISG15, which also lacks anti-SARS-CoV-2 activity (new Supplementary Movie 19 vs. 11, Supplementary Figure 41). Human ISG15 was the weakest antiviral ISG15 and is known to be a good substrate for PLpro cleavage, making it susceptible to viral inhibition. In contrast to *R. trifoliatus,* ISG15 of *R. yonghoiseni* had a very different predicted structure when bound to PLpro, binding residues away from the catalytic pocket, which is suggestive of inefficient PLpro cleavage (Supplementary Movie 15). Thus, identifying which specific residue combinations are required for modifying the structure of *R. trifoliatus* ISG15 to mimic that of human is an interesting topic for a future investigation that focuses on functional ISG15 differences between closely-related bats.



R. yonghoiseni **R. trifoliatus**

3.Related to line 427-430: Since the Cys78 of ISG15 is absent in rhinolophid and hipposiderid bats, why not delete Cys78 in human ISG15 rather than mutating it to validate the function of Cys78? In Figure 4G, restoration of this cysteine in R.affinis ISG15 did not affect its antiviral effect, suggesting that Cys78 may not be important for the antiviral function of bat ISG15. In contrast, mutating Cys78 in human ISG15 significantly reduced SARS-CoV2 replication. Why is this?

This is an excellent point and we have now tested the human ISG15, where Cys78 is deleted. As in previous points, all experiments were performed as initially described, using cells of the same passage number to generate stable cell lines to ensure consistency.
As shown in a new Figure panel 4D and E, removing Cys78 in human ISG affects the antiviral activity similar to the C78A mutation for VSV and IAV, increasing virus production compared with wildtype human ISG15. For coronaviruses, Cys78 mutants generally decrease virus production; however, there are differences between C78A and Cys78 deletion mutants. For both HCoV-229E and SARS-CoV-2 (Fig. 4F, G), only the C78A but not Cys78 deletion behaved differently compared with wildtype human ISG15. Similarly, addition of the Cys to *R. affinis* ISG15 increased its antiviral activity against HCoV-229E but did not affect the activity against SARS-CoV-2.
Importantly, we now experimentally confirm that deleting or mutating Cys78 in human ISG15 as well as restoring Cys78 in *R. affinis* is crucial for the formation of stable ISG15 dimers (please see point 4 below). Therefore, the variable antiviral effects of different Cys78 mutations is not due to differences in dimerization. Instead, these differences are likely caused by differences in the strength of ISGylation and the ISGylated target proteins (Fig. 4C, H-K), which may be affected by differences in the shape of the ISG15 protein. These differences in shape may be caused by mutations in Cys78 as well as other positions located at the surface or in key binding regions, as indicated by our AlphaFold2 modeling (new Supplementary Figures 24 and 41). We now discuss ISG15 shape differences and ISGylation differences as a contributor for different antiviral activities, combined with potentially differential binding to SARS-CoV-2 PLpro in the Discussion:
“First, while we confirmed that Cys-lacking ISG15 does not form stable dimers, our mutation experiments show that this residue has other unpredictable effects on antiviral activity of ISG15. For example, with IAV and HCoV-229E, we observed opposite effects when introducing an equivalent Cys mutation in human or R. affinis ISG15. Likewise, deleting Cys78 or mutating it to Alanine often had differential effects, exemplified by SARS-CoV-2 where C78A but not the Cys78 deletion conferred human ISG15 an antiviral ability. Therefore epistatic interactions with other ISG15 residues also modulate ISG15 shape and function.”

4.In Supplementary Figure 19-22, the range of topologically distinct conformations predicted by AlphaFold2 does not necessarily indicate an unstable structure, but rather reflects the potential flexibility and adaptability of a protein under different conditions. To determine if homodimers of bat ISG15 proteins with Cys78 deletion differ from human ISG15, additional evidence is needed, such as ISG15 dimer detection by SDS-PAGE of both bat and human ISG15 in non-reducing conditions (without 2-Mercaptoethanol) (like in PMID 18606809).

We agree with this comment and a similar point was raised by Reviewer 4. First, we have now conducted additional MD simulations and computational analyses, showing that “the Cys78-lacking ISG15 dimer of both bats appeared more unstable. They adopted topologically distinct conformations that strongly deviated from each other and from the initial AlphaFold2 structure

(Supplementary Figures 27-30, Supplementary Movies 1-9).” as described in the revised main text. Please see also our responses to Reviewer 4 and the new Supplementary Figures and Movies.

Second, following the suggestion, we have attempted to use a native PAGE system that preserves the native conformations of proteins, thereby enhancing our ability to detect homodimers. Since ISG15 has a small size (17 kDa) and specific molecular characteristics, we tried a variety of methodologies to optimize the detection and analysis of ISG15 protein and its monomers and dimers. Initially, the utilization of commercially available native PAGE gel loading dye, electrophoresis buffer, and precast gel was attempted. However, this approach proved unsuccessful, likely due to the incompatibility with the isoelectric point of human ISG15 (6.83) in our system, as the gel's pH was 6.8. Recognizing this limitation, a customized gel system was developed with a separating gel pH of 8.8, theoretically enabling ISG15 migration towards the cathode. Despite these adjustments, the clarity of separation between homodimers and isoforms remained insufficient, likely attributable to the small size of ISG15. Therefore, to detect potentially weak dimers that may form only transiently, we investigated protein conjugations using both DSP (Di(N-succinimidyl) 3,3'-Dithiodipropionate) (Aladdin, PubChem CID: 93313) and BMH (1, 6-Bis(maleimido)hexane) (Aladdin, PubChem CID: 20992). DSP, a cross-linking reagent, forms amide bonds with molecules containing primary amine groups, while BMH, a homobifunctional sulfhydryl reactive reagent, was employed to detect dimer formations.
Using this system, we were able to successfully detect both stable dimers for ISG15 proteins with Cys78 and protein conjugation as ISGylated proteins. We show that in the absence of Cys78, as in the two human Cys78 mutants and in wildtype *R. affinis*, essentially no stable dimers are formed (Fig. 4B,C, Supplementary Figures 38-40). Additionally, we show that ISG15 of a non-horseshoe bat containing a natural Cys78 (*Mops condylurus*) forms stable dimers. This confirms our MD simulation results and demonstrates that the presence or absence of Cys78 determines whether ISG15 can form stable dimers.

In addition, to overcome differential antibody binding efficiency between species as a factor influencing these results, we have since added a Myc tag to twelve ISG15 constructs. This allows us to use a highly-specific and sensitive Myc antibody to detect only the overexpressed ISG15 and not endogenous ISG15. We show that this does not affect dimerization or conjugation as Myc construct conjugation matches well the IRES-mCherry reporter constructs (Fig. 4B, Supplementary Figure 43). We thank the reviewer for this important suggestion which allowed us to explore deeper the relationship of ISGylation and protein conjugation, in addition to dimer formation.

5.Why are the titers of Homo sapiens-C78S and Rhinolophus affinis-S77-78C missing in Figure 4G? Please include these.

We thank the reviewer for pointing out this omission. Due to experimental constraints in the BSL3 laboratory, not all constructs were synthesized for the experiments requiring SARS-CoV2 infections. While we now test a total of 17 constructs with four different viruses, we did not test all mutant constructs with SARS-CoV-2.
We have now additionally tested the human ISG15 Cys78 deletion construct (point 3) against SARS-CoV-2 (Fig. 4G). For consistency, we now present only the two human ISG15 mutants (C78A, ΔC78) and the *R.affinis* ISG15 S77C mutant in Figure 4. Since *R. affinis* S77-78KC behaves very similarly to S77C, we show the S77-78KC results in the Supplemental Figures only.

6.To exclude the effects of endogenous human ISG15, the authors should conduct experiments overexpressing bat ISG15 in ISG15 knockout HEK293 or A549 cells. Additionally, it is currently unknown whether the ISG15 antibody used in this study is effective in detecting bat ISG15. For example, the ISG15 blots in Supplementary Figure 27 do not show any apparent differences between the transfection of empty vector and bat ISG15, indicating that either the overexpressed ISG15 levels are very low or the antibody is ineffective. This is a major concern. Given the uncertain expression levels of bat ISG15 (compared to human ISG15), the related antiviral functional assays against viruses and pro-inflammatory functions of bats ISG15 may not be entirely convincing.

We agree with this concern regarding antibody compatibility. As described in comment 4 above, to address this, we first purchased a second ISG15 antibody, which showed a better detection of ISG15 across species, though may still exhibit a bias. Therefore, to fully address this point and eliminate this bias, we added a Myc tag to all ISG15 constructs. As the Myc tag antibody is highly specific and sensitive, this enables precise detection of Myc-tagged ISG15 and removes antibody variability as a confounding factor in our cross-species experiments.

We also generated ISG15 KO HEK293 cells (shown in a new Supplementary Figure 32) and repeated the infection experiments with myc-tagged constructs, which showed similar results as previously observed (new Fig. 4D-G). The results in the previous Supplementary Figure 27 were likely related to detection differences with the previous antibody. With the Myc antibody, we easily observe myc-specific ISG15 monomer, dimers and conjugated proteins, as well as secreted myc-ISG15 in the supernatants of cells. This is well above the background of empty vector and only myc-ISG15-specific bands were considered when calculating conjugated or dimer ISG15 (e.g. Fig. 4B, Supplementary Figure 38). Given this new labeling for ISG15 we can successfully overcome any bias between species or detection of endogenous protein.

7.Figure 4H: It is somewhat hard to believe that there is no free ISG15 in all bats. As mentioned above, there is a major concern about antibody compatibility. In addition, the authors need to confirm whether the human ISGylation machinery works for bat ISG15. Therefore, the authors should compare the bat ISG15 conjugation capacity in ISG15 knockout background. In these assays, bat ISG15-AA mutant (LRGG mutated to LRAA), which cannot be conjugate to substrates, should be included as a negative control.

We thank the reviewer for raising these points.
First, regarding free ISG15 of bats, we did observe slightly more free bat-ISG15 when using the second ISG15 antibody; however, our biggest concern was that there was still some degree of bias and it was not possible to disentangle if the bias was consistent across all forms of ISG15 (free, dimer, conjugated). Therefore, we have now used Myc-tagged bat ISG15 constructs in HEK293-ISG15 KO cells (please see comments 4 and 6 above). These experiments show that while there is definitely free monomeric ISG15 for the *R. affinis* bat, there is more conjugated *R. affinis* ISG15 (Fig. 4C).

Second, to confirm that the human ISGylation machinery works for bat ISG15, we include full Myc-ISG15 Western blots in Fig. 4B and Supplementary Figures 38 and 39. They show efficient conjugation of bat ISG15 molecules, via the presence of specific myc-ISG15 bands or a smear of ISGylated proteins after induction, both for human and for multiple bat ISG15s. Additionally, as only

the overexpressed ISG15 constructs but not endogenous human ISG15 is myc-tagged, only proteins ISGylated by the ISG15 constructs are then detected. We also validated this in ISG15KO HEK293 cells with similar results. Together, this confirms the human E1-E2-E3 machinery is fully capable of conjugating bat ISG15s to human proteins

To further test the requirement for conjugation in SARS-CoV-2 infection, we followed the reviewer's recommendation and mutated the LRGG ISGylation motif to LRAA in two rhinolophid species (*R. affinis* and *R. yonghoiseni*) and performed SARS-CoV-2 infections, the family of viruses associated with rhinolophid immune signatures. As shown in Figure 5D, mutating LRGG in ISG15 leads to a drastic decrease in antiviral capacity. As the flexible tail of ISG15 is not predicted to impact structure in any other way, and the cleavage site for PLpro is still present in the mutated versions, this functional difference is likely caused by the absence of conjugation ability.

8.Given the potential differences in the ISGylation machinery between bats and humans, the authors should express in bat cells two representative bat ISG15 proteins with Cys78 deletion, and also validate whether restoring Cys78 can alter their ISGylation and secretion capacity.

We thank the reviewer for this suggestion. As requested, using the same constructs with and without Cys78 used in the experiments with human cells, we now expressed these Myc-tagged constructs in RsKT kidney cells from the horseshoe bat *R. sinicus*. We observed a conjugation efficiency in bat RsKT cells that is similar to human cells (new Supplementary Figure 40), indicating successful utilization of bat machinery to conjugate human ISG15, regardless of Cys78-status. As we have previously shown that USP18 in bats is strongly polyI:C-inducible, there would be a constant balance of conjugation and de-ISGylation, post-polyI:C treatment. This may explain the lower observed levels of conjugation seen in this cell line. Despite high expression and detection of the Myc-specific constructs, we fail to detect secreted ISG15 from human or bats from these RsKT cells. Whether the molecular machinery required for secretion in this cell line is intact requires further investigation. We do however show conjugation of human and Cys78-deleted ISG15 constructs in bat cells (Supplementary Figure 40).

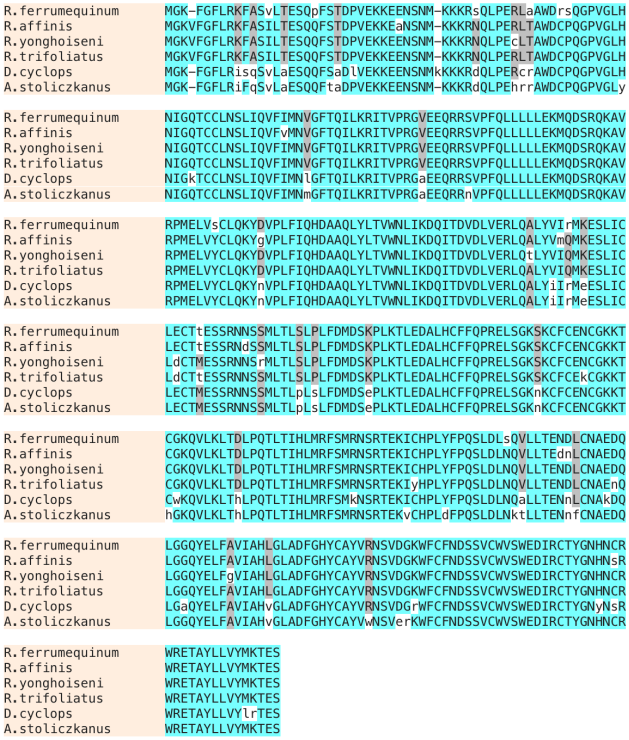
9.In the experiments shown in Figure 4H (right blots), wildtype ISG15 of human and Raff should be included as well. Otherwise, the secreted ISG15 levels of the mutants and wildtype ISG15 on different blots cannot be compared.

We fully agree and the new Figure 4H-K panel shows the wildtype ISG15 of human and *R. affinis*, together with the Cys mutations and restorations, quantified, from the same blot.

10.Ubiquitin-specific protease 18 (USP18) is the primary ISG15-specific protease that counteracts ISG15 conjugation. Does it undergo positive selection in bats? Since the conjugation of overexpressed bat ISG15 in human cells may differ from that in bat cells due to differences in human and bat USP18, it is suggested to synthesize a representative bat USP18 and check if it functions normally in deISGylating bat ISG15 when compared to human USP18.

We thank the reviewer for raising this interesting point. We agree that de-ISGylation, mediated by USP18 or other factors, is important to regulate ISG15's activity. As suggested, we inspected USP18

and found that the protein sequence is overall highly conserved between rhinolophid and hipposiderid bats.



There is also no evidence for positive selection in any bats included in our screen (the lowest aBSREL P-value for any bat branch is 0.092). Given the species-specific effects of ISG15, a thorough investigation of the role of USP18 would ideally require a comparison of USP18 from all studied bats, presumably together with their E1-E2-E3 ligases. To investigate the overexpressed USP18 and ISG15, one would need to first generate USP18 and ISG15 double knockout *R. sinicus* cells. Given the complexity of these experiments and given that USP18 of a single chosen bat may not be representative, we believe that this is an interesting topic for a future study.

Instead, to study deISGylation, we decided to focus on the SARS-CoV-2 encoded protease PLpro that is known to de-ISGylate IRF3, MDA5 and other important factors, which contributes to immunopathology. Hence, we investigated whether rhinolophid ISG15 has a higher resistance to PLpro-mediated ISGylation.

PLpro of SARS-CoV-2 was previously shown to efficiently cleave human ISG15 to evade its antiviral functions. We confirmed PLpro's effect on human ISG15, as PLpro increased the amount of ISG15 that is secreted from the cell. Interestingly, we also found that *R. affinis* ISG15 is a poor substrate for PLpro cleavage, while ISG15 of *M. condylurus*, a bat quite distant from other horseshoe bats, was very efficiently cleaved by PLpro. This suggests the evolutionary signature for ISG15 evolution may be matched to coronavirus sensitivity.

Additionally, we investigated the influence of the presence and absence of Cys78 on cleavage by PLpro and subsequent ISGylation (new Fig. 4K). Furthermore, Alphafold2 modeling using the newly published crystal structure of PLpro, but substituting human ISG15 for several bat ISG15's, revealed subtle differences in the contact residues between different bat species. Additionally, we show that

both human and bat ISG15's can be conjugated in bat cell lines, showing consistency and conservation amongst the E1-E2-E3 ligases. Small differences in conjugation efficiency were observed between human and rhinolophid bat ISG15 constructs in *R. sinicus* RsKT cells, induced with polyI:C and expressing endogenous USP18 (new Supplementary Figure 40). We believe this additional data addresses the reviewers concerns, yet additionally provides insight directly relevant to viral infection and evolution of host genes against pathogens.

11.In lines 417-418, the authors state that the viral N protein was significantly reduced by human and R. ferrumequinum ISG15 overexpression. However, in Figure 4H, no obvious difference in HCoV-229E N protein was shown when human and bat ISG15 were overexpressed. Why is this?

The differences observed in the previous Supplementary Figure 28 were rather subtle (7000 vs. 10000 mean fluorescent intensity). Due to the sensitivity of FACS it was easy to observe a small but significant difference that may be difficult to observe on a western blot. To overcome this confusion in the results, we now focused on virus production in the supernatant (rather than mean fluorescent intensity as a surrogate for virus replication inside the cell), as this is the end result of a successful infection. We now display the TCID50 result (relative to the empty vector control) for HCoV-229E virus production in the main figure (Fig. 4F, 5B), and have consistently used a measure of virus production between viruses for a better comparison. We now only use VSV (in the Supplementary Figures 33-35) as an example to highlight how differences in virus entry, replication and cell proliferation are a result of species-specific ISG15 differences, yet may not necessarily correlate with final virus production (i.e. initial defects in entry or early-stage replication can be overcome as viral production reaches high levels and overwhelms the cell).

Minor points:

1.Supplementary Figure 27 and Supplementary Figure 30: These blots are of low quality and should be repeated and improved.

These westerns have been replaced as we are now utilizing myc-tagged constructs for clarity.

2.Molecular weight markers should be included in all blots.

We have included molecular weight marker for all blots where ISGylation is indicated and show the complete western for clarity in the Supplementary Data. For presentation purposes only, some cropped western blot panels, where a range of markers are used, do not display the marker; however, all uncropped and unmodified western blot images, including molecular weight markers, are shown in the Supplementary Data.

3.In Supplementary Figure 9, "(A)" and "(B)" are missing from the figure.

We thank the reviewer for noticing that. It is now fixed in the reordered Supplementary Figure 11.

4.In Supplementary Figure 10, "(B-I)" should be changed to "(B-E)".

This is also fixed in the reordered Supplementary Figure 15 legend.

Referee #4 structural modeling

In their manuscript, Morales and Dong et al. present new genome assemblies of ten bat species and, by analysis of positive selection across these genomes and those of other mammalian species, conclude that the positive selection in the immune genes is highest in bats. They review the current knowledge about the immune roles of the selected genes that may help the reader interpret the findings. Then, they analyze in detail one of the genes, ISG15, in which a cysteine 78 residue is deleted in some of the bats. They note that in other species, Cys78 "is required for the formation of stable ISG15 homodimers and its extracellular cytokine function", and that "mutating Cys78 enhances ISGylation, likely because dimerized ISG15 is not usable for ISGylation". They thus start the ISG15 analysis with structural modeling using AlphaFold, I-TASSER, and molecular dynamics (MD) to conclude that even though AlphaFold predicts dimeric structures for bat ISG15s, the dimers are unstable, compared to the stable human ISG15 AlphaFold dimeric model. They follow by an experimental analysis in cell culture to investigate the capacities of bat ISG15s to restrict infection of four different viruses. They find that different ISG15s have various effects on restricting the viruses, but the results depend on the bat and virus species, so other factors must also play a role. They finish by confirming that bat ISG15s that lack Cys78 are not secreted from the cells infected with coronavirus as is the human Cys78-containing ISG15.

Overall, the manuscript is an exciting and accessible read, even for a general audience from other fields. The finding that immune genes are positively selected in bats and the presented specific list of the genes addresses the general curiosity about mechanisms behind bats being such a successful reservoir of various viruses. However, focusing my revision on the structural modeling part of the manuscript, I have major comments about this part, which in its current form does not support the conclusion that the human ISG15 dimer is stable while the non-cysteine bat variants are unstable:

> We thank the reviewer for these kind words and the comments that helped us to improve the structural modeling part of the manuscript.

1) The confidence scores of the AlphaFold models, especially those shown in Supp. Figures, should be shown. These should include at least the global pTM, ipTM, the per-residue pLDDT (mapped to the models using the standard AlphaFold pLDDT coloring scheme), and, for the dimeric models, the PAE plots.

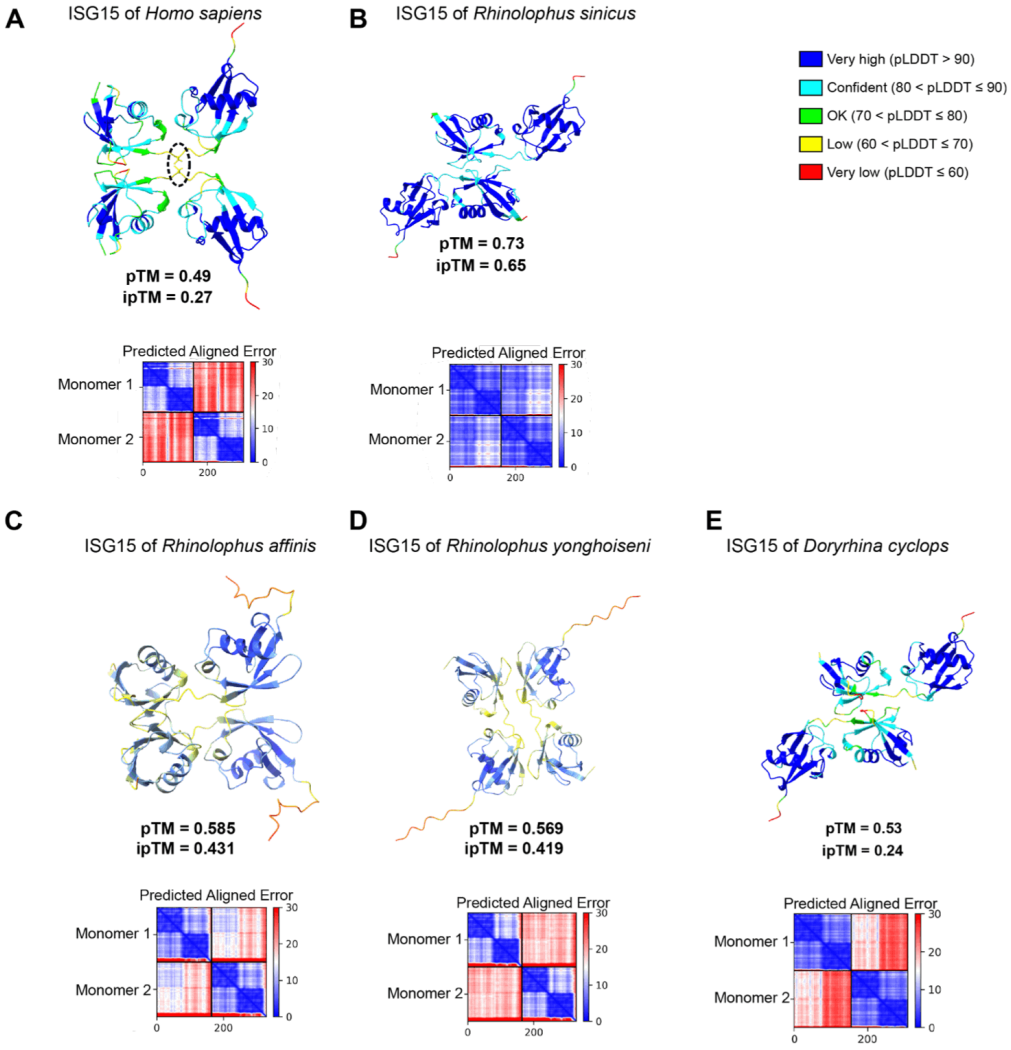
> We now added a new Supplementary Figure 24 that shows pTM (predicted Template Modeling), ipTM (predicted interface Template Modeling), pLDDT (predicted Local Distance Difference Test), and the PAE (Predicted Aligned Error) confidence scores, as reported from the ColabFold pipeline, utilizing the standard AlphaFold2 color palette and scaling. We discuss the implications of these scores and why MD simulations were necessary in the next response.

2) Are the AlphaFold interaction scores (ipTM, PAE) high for the bat dimers? If so, how the claimed instability of MD simulations could be explained? Do the authors mean that the AlphaFold dimers false positives in these cases? This should be explained to the readers.

> We show the new Supplementary Figure 24 below, which now includes these confidence scores. As detailed in the legend of the figure, AlphaFold predicts the monomer structure with a high confidence. However, the confidence scores for ISG15 dimerization contradict experimental results for human (false

negative, as AlphaFold fails to predict a stable dimer). We also added models for *R. trifoliatus* and *R. yonghoiseni* now. For ISG15 of the four bats (*R. sinicus, R. trifoliatus, R. yonghoiseni* and *D. cyclops*), which all lack Cys78, the confidence scores for the dimeric interface (ipTM) differ substantially, which could potentially indicate a false positive for *D. cyclops* ISG15. We therefore were cautious to not draw strong conclusions from the AlphaFold predictions, and conduct additional molecular dynamics simulations to estimate whether the AlphaFold structures remain stable over time.

We added to the main text:
"As we found discrepancies between the dimeric interface confidence scores generated and previous experimental studies for human ISG15 [116,117], as well as predicted variations in stability between the bat species (Supplementary Figure 24), we evaluated the stability of predicted dimers by conducting three replicate molecular dynamics simulations …"



Supplementary Figure 24. ISG15 dimer structures of five species estimated with AlphaFold and the reported confidence scores.
(A) Human ISG15. The pTM (predicted Template Modeling) score of 0.49 is close to the commonly used TM cutoff of 0.5. Furthermore, the pLDDT (predicted Local Distance Difference Test) scores along the structure of each monomer indicate a high or acceptable confidence. Together, this indicates that the overall quality of the structural ISG15 monomer model is

3) I do not understand why the I-TASSER model was used to predict the structure of the human ISG15 monomer (Supp. Fig. 22), as crystal structures seem to be available (PDB IDs: 1Z2M, 7S6P). The supp. fig. 22 should either include the panel comparing the I-TASSER model to the crystal structure (and have all-atom RMSD in the legend) and explain the reason for modeling to the reader or be redrawn to use the crystal structure.

I-TASSER was initially used before we conducted AlphaFold and MD simulations.
We agree with the next comment of the reviewer that AlphaFold is often more accurate, and the confidence scores indicate that the monomer is predicted well. We therefore removed the I-TASSER model from the manuscript.

4) If it is necessary to use a theoretical model of the human ISG15 monomer for some specific reasons, why use I-TASSER instead of the usually more accurate AlphaFold? The supp. fig. 22 should either include the panel comparing the I-TASSER to AlphaFold – as the current baseline modeling program – or be redrawn to use the AlphaFold model.

We removed the I-TASSER analysis, as mentioned above.

5) The use of MD to validate AlphaFold models is very interesting. Has this approach been systematically validated yet, though? Do we actually know yet what a more accurate method for assessing the confidence of AlphaFold dimers in general is – AlphaFold native scores or stability in MD simulations?

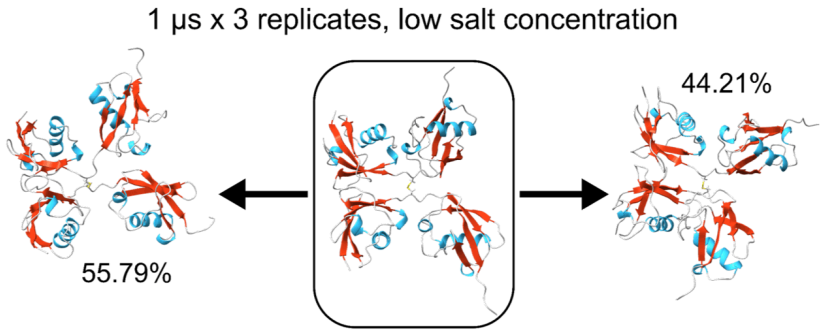
We are not aware of any study that has systematically compared the predictions of AlphaFold with those of molecular dynamics simulations, specifically for evaluating the stability of dimers (or multimers). However, given that AlphaFold ultimately produces static structural models and cannot provide insights on the dynamics of the structure in solution, we expect molecular dynamics simulations to be more reliable for this purpose. This is supported by Supplementary Figure 24 (comment 2 above), where we note that AlphaFold predicted that human ISG15 forms weak dimers (if at all), contrary to experimental evidence.

6) The analysis of the MD simulations is limited to clustering MD conformations and showing pictures of cluster representatives. This makes it difficult to appreciate whether the simulations support the claim about the bat ISG15 dimers being unstable and human ISG15 dimer stable. In fact, for the bat examples (Supp. Figs. 19 and 20), the actual dimeric interface seems to be preserved in all representatives while only the outer non-interacting domains are flying around. On the other hand, for the human ISG15 (Supp. Fig. 20), even the dimeric interface seems to change, although it is hard to judge as the models are shown in different orientations (the disulfide bridge remains, but it is obvious as MD cannot break bonds). A quantitative analysis is absolutely necessary to show whether dimeric interfaces are more stable in the human ISG15, possibly including positional root mean square deviations, a fraction of native contacts from the initial model across the simulation, and overlays of the structures superposed on the dimerization interface across the simulation time. Perhaps the following publication could provide some guidelines on how to perform a convincing analysis: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8444332/

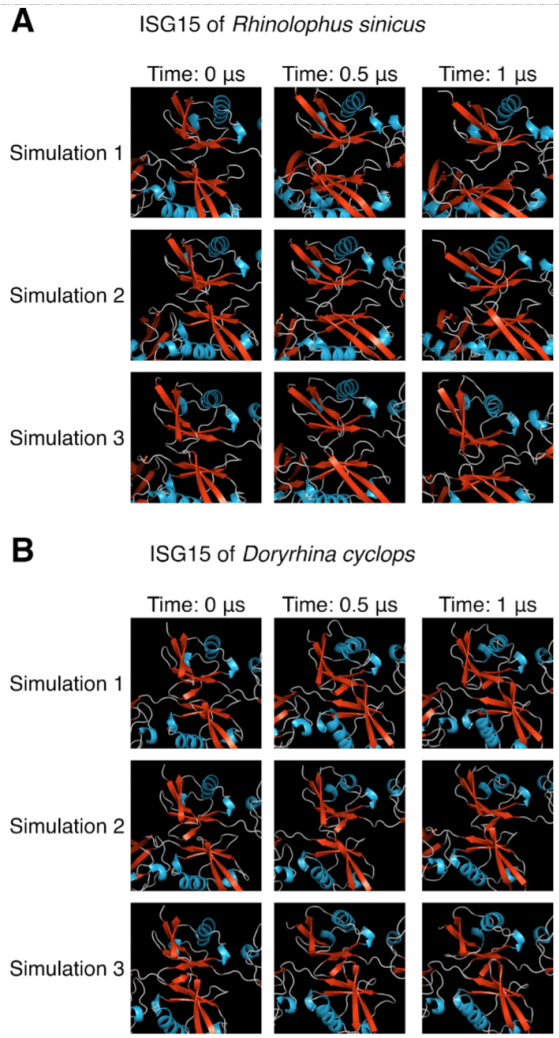
We thank the reviewer for these helpful suggestions. Similar to the procedure described in (Jandova et al. 2021), we analyzed the fraction of native hydrogen bonds between the two monomers over the course of the simulation, which is described in detail below (point #7).

We have added a new Supplementary Figure 30, showing 3 snapshots of the three simulation replicates for ISG15 of bats. Furthermore, we added Supplementary Movies of all three simulations for the three species (please see also point #13 below).

Based on the conformation analysis (Supplementary Figure 27) and on the simulation movies, the overall structure of the human dimer is preserved. In particular, while the monomers rotate slightly, resulting in highly similar major conformations, the disulfide bond (which cannot be broken in the MD simulation; please see point #9 below) is sufficient to result in a stable dimeric interface (we added this point to the legend of Supplementary Figure 27).
In contrast, the conformations of the bat ISG15s strongly differ from the initial AlphaFold structures as simulation time increases, including in the dimeric interface. To make this clearer, we have now added a new Supplementary Figure 30 that shows the interface between the ISG15 monomers of the two bats at three representative simulation time points. These results indicate that the bat dimers that AlphaFold predicted are not stable, given that they undergo pronounced conformational changes, including in the dimeric interface. Consistent with this, different interface conformations are observed in our three replicate simulations (please see also the Supplementary Movies 1-9).



Supplementary Figure 27. Conformations of putative dimers of human ISG15.
The dimer that we estimated with AlphaFold is circled, whereas the two other dimers are the two major conformations, observed during molecular dynamics simulations (see Methods). Both major conformations are very similar to the AlphaFold predicted conformation. Importantly, while the monomers slightly rotate around the disulfide bond, the Cys78-mediated disulfide bond is sufficient to maintain a dimeric interface with a highly similar conformation, indicative of a stable dimer (see also Supplementary Movies 1-3). These results are consistent with experimental studies [10,11], with the molecular dynamics simulations for human ISG15 serving as a positive control since the disulfide bond cannot be broken in the simulation. Helices are shown in cyan, whereas β-strands in red. The percentages correspond to the proportion of each conformation across the simulations. The figure was rendered with UCSF ChimeraX v.1.2 [12].

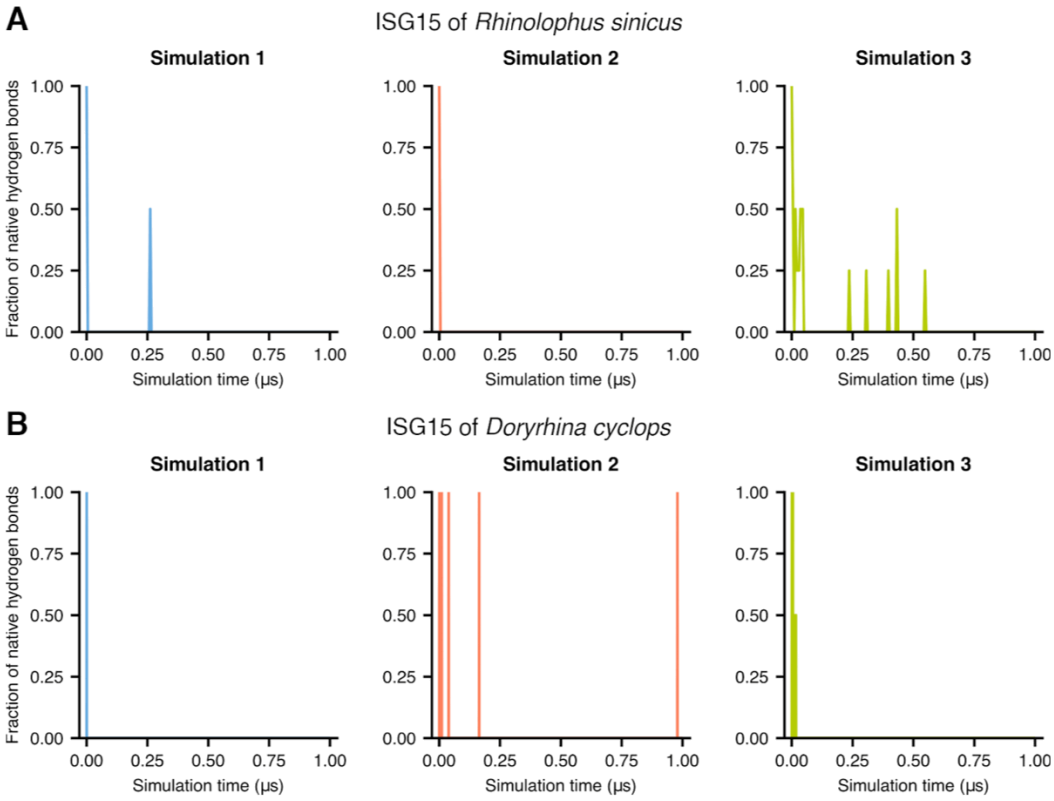


Supplementary Figure 30. Snapshots of the dimeric interface of the ISG15 of *Rhinolophus sinicus* (A) and *Doryrhina cyclops* (B) during molecular dynamics simulations performed with a low salt concentration.
While the three simulations initially exhibit highly similar dimeric interfaces, because the starting point is the same AlphaFold predicted dimer after energy minimization and equilibration, the interfaces increasingly deviate from each other as time increases, indicative of an unstable dimer. See also Supplementary Movies 4-9. The figure was rendered with PyMOL v. 2.5.0 [13].

*7) Related to the above, using whole-dimer RMSD for the comparison and clustering is not informative for the questions asked about the stability of the interfaces – if non-interacting domains are very variable, the RMSD and global variability will be high even if the dimeric interface was preserved. The clusters will represent the variability of those other domains rather than the interface. The analysis should focus on the *interface* RMSD and interface contact analysis.*

Given that the interface changes considerably across simulation time, as shown in the snapshots and movies (comment 6 above), we did not conduct an interface RMSD analysis. Instead, we followed the Reviewer's advice (Jandova et al. 2021) and performed a quantitative analysis where we examined the

fraction of native hydrogen bonds between ISG15 monomers for the two bat ISG15s over simulation time:



Supplementary Figure 31. Fraction of native hydrogen bonds between ISG15 monomers of *Rhinolophus sinicus* (A) and *Doryrhina cyclops* (B).
For both species, very few hydrogen bonds were initially present at the dimeric ISG15 interface at the beginning of the simulations. However, these bonds are quickly broken, reflecting the conformational changes between the two monomers, which is consistent with *R. sinicus and D. cyclops* ISG15 not forming stable dimers.

The results show that the few hydrogen bonds that were present in the beginning of the production phase of the molecular dynamics simulations are quickly broken and are very rarely formed again during the course of the simulations. This is consistent with the findings of our analysis of representative conformations.
We added this to the main text:
“We first used structural modeling to explore whether Cys78 deletion affects homodimer formation of bat ISG15. We hypothesize two alternative outcomes. A stable homodimer will not be formed if we delete Cys78 due to the removal of the disulfide bond. Alternatively, stable homodimers might still form if the dimeric interface is maintained by compensating sets of hydrogen bonds. To test these hypotheses, …
Furthermore, a quantitative analysis of native hydrogen bonds throughout simulation time showed that hydrogen bonds that were initially present between ISG15 monomers of the two bats were quickly broken and only reappeared transiently or not at all (Supplementary Figure 31). Together, hydrogen bonds are likely not sufficient to maintain a stable dimeric interface, supporting our first hypothesis and suggesting that Cys78 deletion in bats results in unstable ISG15 homodimers.”

8) The quantitative analysis should also consider the AlphaFold confidence scores. For example, suppose the dimeric interface is composed of two parts, one well supported by the PAE and pLDDT scores and another poorly scored (as is frequent in AlphaFold models). In such case, it should be checked whether the confident part remains stable while only the non-confident one is variable (in other words, the interface RMSD or contact analysis including the non-confident part would give artificially high variability).

The reviewer raises a very good point. For human and *Doryrhina cyclops* ISG15, our Supplementary Figure 24A,E (above in comment 2) shows that the pLDDT scores are low across the entire interface and that PAE values are high for the entire dimer. Thus, for these two species, there is no part of the dimeric interface that is predicted to be stable. In contrast, for *Rhinolophus sinicus* for example, the entire interface has confident pLDDT scores and low PAE values.

9) The quantitative analysis may reveal that the human dimers do not have more stable interfaces than bats except for the disulfide bridge. Still, as the disulfide bond cannot be broken in MD simulation, the human ISG15s remain bound. MD is not necessary to conclude that two proteins connected with a disulfide bond would be stable.

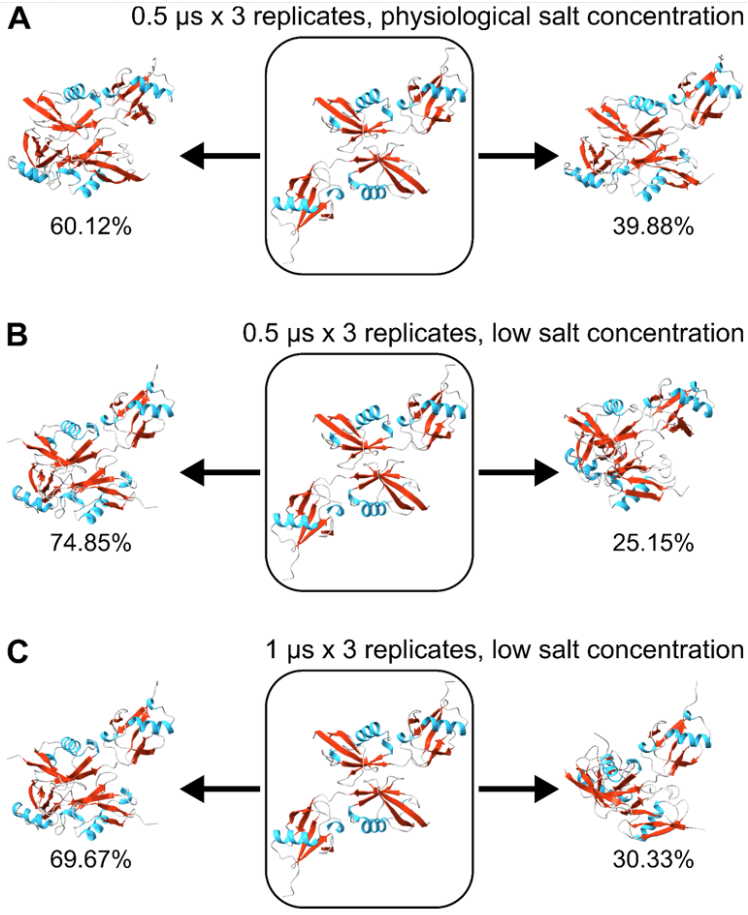
We are aware that the molecular dynamics simulations would not break the disulfide bond, and we agree with the reviewer that the stable human ISG15 dimeric interface is primarily maintained by the Cys78-mediated disulfide bond. Indeed, we did not observe the formation of stable hydrogen bonds in the dimeric interface over the course of the simulations.

We would like to note that our expectation was indeed that the human dimer primarily depends on the disulfide bond and not on interactions among other residues, as experimental evidence has shown that mutating Cys78 is sufficient for disruption of human ISG15 dimers (Okumura et al. 2008; Napolitano et al. 2018). Nevertheless, we chose to conduct simulations also for the human ISG15 so that they could act as a positive control. We explain this and provide more details about the stability of the dimer interface and consistency with experiments in the legend of Supplementary Figure 27:
"... Both major conformations are very similar to the AlphaFold predicted conformation. Importantly, while the monomers slightly rotate around the disulfide bond, the Cys78-mediated disulfide bond is sufficient to maintain a dimeric interface with a highly similar conformation, indicative of a stable dimer (see also Supplementary Movies 1-3). These results are consistent with experimental studies [10,11], with the molecular dynamics simulations for human ISG15 serving as a positive control since the disulfide bond cannot be broken in the simulation."

10) Na+ and Cl- ions were used only for neutralizing the system. Shouldn't the simulations be run in the usual closer-to-physiological 150 mM salt concentration? Could the reduced salt concentration affect the stability of the dimers in the simulations?

We thank the reviewer for raising this point. A hypothetical weak dimer would be increasingly destabilized by the addition of salt because salt ions would compete with the interactions that would previously stabilize the weak dimer. Our simulations in the submitted manuscript used a concentration required for neutralizing the system, as stated in the methods. To explore the effect of higher salt concentrations, we have now conducted three additional replicate simulations of the *Rhinolophus sinicus* ISG15 at physiological salt concentration. We used half the duration of our other simulations

(i.e., 0.5 µs), which was sufficient to compare results with the low salt concentration simulations for the same duration. Increasing salt concentration to 150 mM (physiological salt concentration) led to qualitatively identical results, which we added to the Supplementary Figure 29. As explained in the legend, the lack of physiological salt concentration did not influence our results.

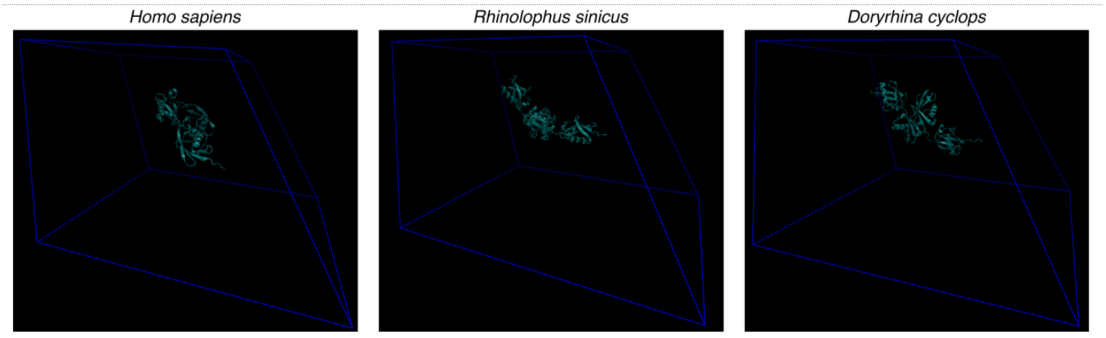


Supplementary Figure 29. Conformations of putative dimers of the ISG15 of *Rhinolophus sinicus*, both at low and physiological salt concentrations

(A-C) Each panel shows the dimer that we estimated with AlphaFold (circled) and other dimers that are major conformations observed during molecular dynamics simulations with different salt concentrations and durations (see Methods). As for *D. cyclops* ISG15, the lack of the Cys78-mediated disulfide bond appears to consistently result in an unstable dimer that adopts two major conformations that differ considerably in the spatial arrangement of the monomers, both from each other and from the starting AlphaFold structure (see also Supplementary Figure 30 and Supplementary Movies 7-9). This result was observed both at physiological (A) and at salt concentrations required to neutralize the system (low salt, panel B) for a comparable simulation time of up to 0.5 µs (panel A and B), indicating that different salt concentrations consistently result in unstable dimers. Comparing low salt conditions for a simulation time of 0.5 vs. 1 µs (panel B and C), we find even larger structural differences between the major conformations, which is consistent with the expectation that as the simulation time increases, unstable dimers would increasingly deviate from the initial structure. Overall, these tests also indicate that our results are robust to variation in the salt concentration used in the simulations. Helices are shown in cyan, whereas β-strands in red. The percentages correspond to the proportion of each conformation across the simulations. The figure was rendered with UCSF ChimeraX v.1.2 [12].

11) I find it odd that the protein distorts so much, but the dimer doesn't fall apart in the bat simulations. This could be either that the dimers are stable after all, the low salt issue over-stabilizing the interfaces, or too small bounding box. For the former two, see comments above, but for the latter, the authors should show a snapshot of the initial system, including lines indicating the simulation box to make sure the padding was big enough.

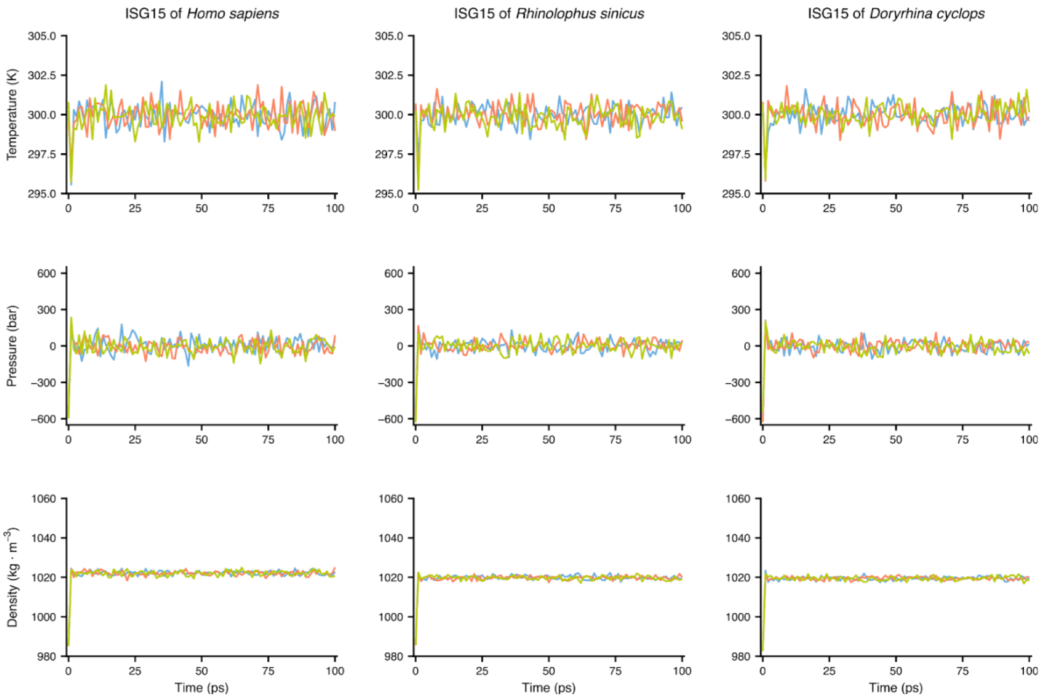
We enforced a minimum distance of 2.5 nm between the protein and each box edge, which can be considered more than enough for preventing any simulation artifacts. As suggested, we have also added Supplementary Figure 25 to better highlight this:



Supplementary Figure 25. Snapshots of the initial simulation boxes.
We enforced a minimum distance of 2.5 nm between the protein and the edges of the box, which is sufficient to prevent simulation artifacts. The panels were rendered with VMD v. 1.9.4a57.

12) The equilibration time seems a bit short - 100 ps - while some studies report longer times ranging from a few nanoseconds to tens or even hundreds of nanoseconds (https://pubmed.ncbi.nlm.nih.gov/17280090/, https://onlinelibrary.wiley.com/doi/10.1002/prot.26161). Although the equilibration time length of time required to equilibrate a protein system in molecular dynamics simulations can vary depending on the specific system being studied, the force field used, and the simulation conditions, could it be that the ISG15 models fall apart due to a lack of equilibration? Perhaps, in this case, a longer equilibration is needed because the starting structures are theoretical models?

We previously wrote in the manuscript that we verified that the equilibration time was sufficient by manually examining how the temperature, pressure, and density of the system change with time; however, we did not show evidence of this. Therefore, we have now added a new Supplementary Figure 26 showing the values of these three variables over time for the three replicate simulations (different colors). The figure shows that the system is already successfully equilibrated within less than 25 picoseconds.



Supplementary Figure 26. Temperature, pressure, and density of the ISG15 systems during the equilibration phase of molecular dynamics simulations with low salt concentration.
Each replicate simulation is shown in a different color. The systems appear to be successfully equilibrated within less than 25 picoseconds (ps). We conservatively set the duration of each equilibration step to 100 ps.

13) Videos of representative simulations should be included in the manuscript supplementary information. They could perhaps show whether the models distort quickly due to the lack of equilibration.

As mentioned above, we have now added a Supplementary Movie 1-9 for each of the three simulations for each of the three ISG15s with the initial (low) salt concentration.

14) The analysis of DDG of mutations in ISG15 using DynaMut doesn't seem to add much to the manuscript and brings additional prediction uncertainty (due to the non-perfect predictive power of the method and because the analysis does not consider the dimeric conformation), but if the authors prefer to keep it, it probably should be redone with the crystal structure rather than I-TASSER model.

We agree with the Reviewer on the accuracy of the model based on I-TASSER, we therefore decided to remove such analyses as they are not adding new information.

15) The Methods section says simulations were run for 1 microsecond, while the text and figures state 3 microseconds. I guess this is just a typo in the methods section?

We apologize for the lack of clarity. We conducted three replicate simulations for each ISG15. The duration of each simulation was 1 µs. Therefore, the total simulation time per ISG15 was 3 µs.
We have made that clear in the main text:
"... we evaluated the stability of predicted dimers by conducting three replicate molecular dynamics simulations in one representative rhinolophid and hipposiderid bat each, for 1 µs per ISG15 (total duration 3 µs, about 550,000 CPU hours) (Supplementary Figures 25-26)."

16) On page 7, lines 219-222 – "42 observed vs. 21 expected selected immune-related genes" in Chiroptera is mentioned as "a larger relative number" compared to "42 observed vs. 24 expected in Afrotheria" – this ratio is almost the same, is there a typo here?

This is not a typo and the numbers are correct, but we agree that "relative number" is not the best description. Instead, describing this difference as a ratio is more informative. The ratio of the observed vs. expected selected immune-related genes in Afrotheria is 1.75 (42 observed vs. 24 expected = 1.75), while the ratio reported in Chiroptera is 2.0 (42 observed vs. 21 expected).

To improve the clarity, we have rephrased this sentence, which now reads:
"Notably, the ancestral chiropteran branch has a two-fold higher number of selected immune-related genes than expected from the length of this branch (42 observed vs. 21 expected), and ancestral branches of all other orders have lower ratios (e.g. ratio 1.75 with 42 observed vs. 24 expected for Afrotheria; ratio 0.95 with 20 observed vs. 21 expected for Rodentia) (Fig. 2C, Supplementary Table 8)."

17) The article already very nicely explains several basics of immunology or explains the modeling methods, making this manuscript accessible to a general reader. Could you also explain the method for detecting positive selection behind the aBSREL software? The "an adaptive branch-site random effects likelihood method implemented in HYPHY" is not clear to a general reader.

We thank the reviewer for pointing out this missing explanation. We have now added a more detailed explanation of the approach implemented in aBSREL that reads as follows:
"To identify genes under positive selection, we used aBSREL 55, an adaptive branch-site random effects likelihood method implemented in HYPHY v.2.5.8 174 that infers the strength of natural selection using the ratio of nonsynonymous to synonymous substitution rates (dN/dS metric). The values of dN/dS are interpreted as sites that experience purifying selection (dN/dS < 1), evolve neutrally (dN/dS ≈ 1), or experience positive diversifying selection (dN/dS > 1). For every phylogenetic branch, aBSREL tests if positive selection has occurred by determining if more complex models that allow a fraction of the codons to evolve with dN/dS > 1 fit the data best. The method uses the Akaike Information Criterion to assess the goodness of fit while penalizing increased model complexities (number of parameters). aBSREL was run in exploratory mode to test all branches and nodes within the phylogenetic tree for 115 mammals. For each gene, aBSREL corrects for multiple testing over all tested branches using the Benjamini-Hochberg procedure."

Point by point response

Referee #1 (Remarks to the Author):

The authors fully addressed all my comments and concerns. I thank the authors for their detailed, thorough response.

> We thank the reviewer for these positive words and are glad all concerns are fully addressed.

Referee #2 (Remarks to the Author):

This is a revised version of a previous manuscript. The authors have addressed all my questions clearly, and I appreciate the thoughtfulness of their response. This is an elegant and well written paper that delves into the complexity of evolution in response to pathogens across the mammalian class, and deeply within the bat lineage. It illustrates the power including high quality reference genomes as part of a much larger resource of both higher and lower quality genomes to explore evolutionary questions. I have no further critiques and recommend it (enthusiastically!) for publication.

> We thank the reviewer for these kind words and for enthusiastically recommending our manuscript for publication.

Referee #3 (Remarks to the Author):

The authors have performed extensive additional experiments to address this reviewer's previous concerns; however, some of the biochemical/mechanistic data are not of sufficient quality and therefore fail to support the major conclusions about the role of deletion of Cys78 in ISG15 in antiviral activity. Therefore, the major concerns about the molecular mechanism presented in Fig. 4 and 5 still remain.

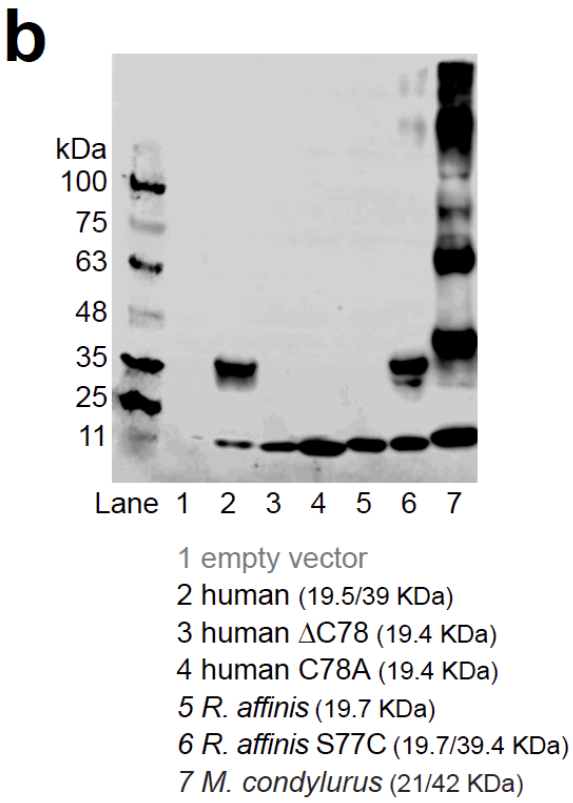
> We apologize for not clarifying in the revised manuscript that we did not use traditional HEK293 cells, but a HEK293-subclone that was selected for high virus production and expresses the entire ISGylation machinery; several concerns were related to this point. We thank the reviewer for highlighting this shortcoming. We now address all points, as detailed below. In particular, we updated the Western blot images to show clear ISGylation bands and demonstrate that our HEK293-subclone cells express the entire ISGylation machinery.

Specific major concerns:

1. In Figure 4B, the expression level of R.a ISG15 in lane 5 is significantly lower compared to that of other species or mutant, making it difficult to accurately compare their antiviral abilities in Figures 4D and 4E.

We agree that the Western blot image to show dimer formation in 4B was not of sufficient quality. We have replaced it with a better example image from the same quantified set. We would also like to mention that all virus infection data was based on FACS-gated normalized reporter gene expression data, such that all cells were equally expressing an IRES reporter (similar RNA levels). All Western blot quantifications were additionally normalized to the ISG15 expression level via myc labeling (also relevant for point 3 below).

The updated Figure 4b panel is:



2. The new results presented in Figure 4 indicate species- and virus-specific differences in antiviral activity that are not solely explained by the presence or absence of Cys78. This suggests that additional mutations in individual bats (as shown in Figure 4A) may also influence ISG15's antiviral activity. Therefore, it is unconvincing that the differences in antiviral ability are primarily due to the Cys78 deletion.

We fully agree with the reviewer and apologize that our text was not sufficiently clear. While our initial hypothesis was that the Cys78 deletion affects ISG15's function, we do not claim that Cys78 solely explains antiviral differences. Instead, our results suggest that epistatic interactions between Cys78 and other residues likely affect ISG15 shape and function. To clarify this important point, we have added additional text in the results, figure legends and discussion, highlighting species-specific functional differences despite a shared Cys78 deletion:

"Notably, we observed significant differences among rhinolophid species that all share the ISG15 Cys78 deletion, indicating that other amino acid mutations alter ISG15 function in a species-specific manner."

"Together, our VSV infection experiments reveal species-specific antiviral differences of rhinolophid/hipposiderid ISG15, not explained by their shared Cys78 deletion, …"

"This suggests that ISG15 antiviral activity is influenced by epistatic interactions between Cys78 and other residues."

"Since the presence or absence of Cys78 has no consistent effect on ISG15's antiviral activity and epistatic effects with other ISG15 residues may play a role, results for *R. affinis* and *M. condylurus* may not generalize to other bats. "

"For IAV, ISG15 of all bats except *R. yonghoiseni* showed antiviral activity compared to the vector control, and many have stronger antiviral activity than human ISG15, but presence of Cys78 did not correlate with antiviral function (Fig. 5a)."

We summarize these findings in the Discussion:

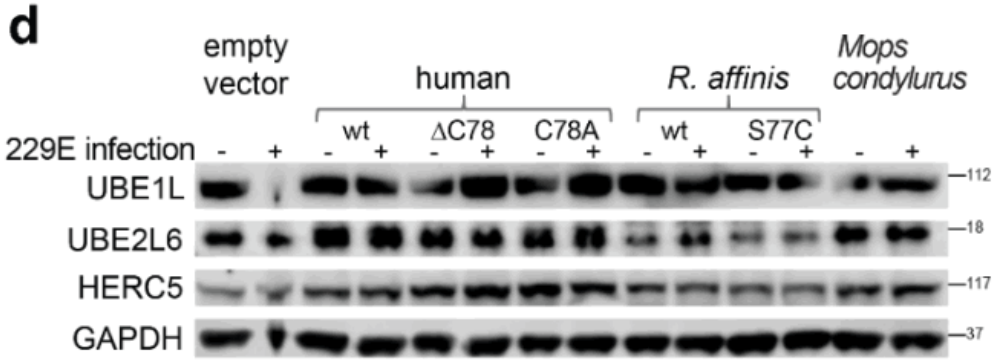
"First, our mutation experiments show that Cys78 has other unpredictable effects on antiviral activity, exemplified by IAV and HCoV-229E, we observed opposite effects when introducing an equivalent Cys mutation in human or *R. affinis* ISG15. Likewise, deleting or mutating Cys78 to Alanine often had differential effects, exemplified by SARS-CoV-2 where C78A but not the Cys78 deletion conferred human ISG15 an antiviral ability. Hence, epistatic interactions with other residues also modulate ISG15 shape and function."

Finally, to explore species-specific differences beyond the shared Cys78 deletion in rhinolophid bats, our new Extended Data Figure 10 shows snapshots of AlphaFold2 models, highlighting species-specific differences in contact residues, particularly in *R. trifoliatus* compared to *R. yonghoiseni*.

3. There are still major concerns about the assays showing ISGylation in the revised manuscript (SI 38 and 39). Overexpression of ISG15 in HEK293T cells alone is insufficient to detect robust ISGylation due to the absence of the complete ISGylation machinery. How could the ISGylation bands predominantly appear between 17-35 kDa? In SI39, the smears observed in lanes 7-14, with or without PLpro, likely reflect free ISG15 expression. Overall, the proposed ISGylation bands appear to be non-specific or possibly due to gel running issues.

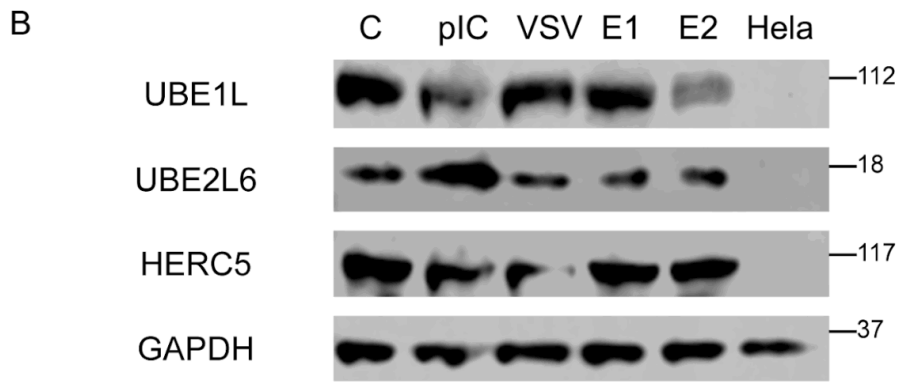
Furthermore, why does lane 6, which overexpresses PLpro, show an enhanced smear compared to lane 5? Consequently, the quantitative data on conjugated ISG15 in Fig. 4 is not convincing and the overall proposed mechanism not sufficiently supported by the new data.

We fully agree that the presence of the complete ISGylation machinery is absolutely key in these experiments. First, we apologize for not clarifying in the main text that our HEK293 cells are a sorted subclone specifically selected for high virus production. This cell line is similar to traditional HEK293 cells, but expresses high baseline levels of ISGs/IRFs and also expresses the entire ISGylation machinery. To demonstrate this, we now validated the expression of the E1, E2 and E3 ligases (UBE1L, UBE2L6 and HERC5) in the exact same samples used for Figure 4h-k. These Western blots are shown in Extended Data Figure 9 with uncropped images available in the Supplementary Data.



d, Western blot of E1 ligase (UBE1L), E2 ligase (UBE2L6) and E3 ligase (HERC5) expression, together with GAPDH in the same samples shown in panel c, indicating sufficient expression of ISGylation machinery in the HEK293-subclone-ΔISG15 cell line.

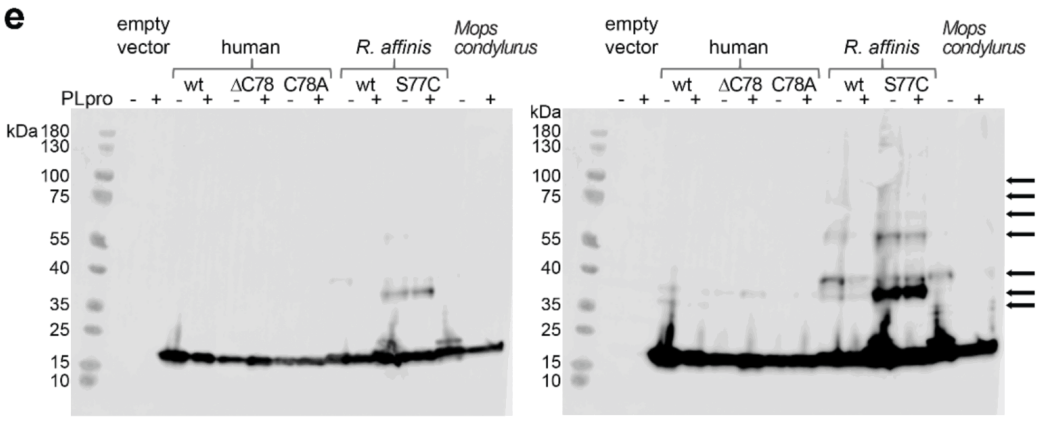
To rule out antibody detection problems, we carefully tested antibody specificity to ensure specific detection of E1, E2 and E3 by (i) low-level overexpression controls, (ii) poly:IC and VSV stimuli, and (iii) by including an ISGylation machinery-deficient cell line like HeLa cells as a negative control (shown in new Supplementary Figure 21B). We would like to mention that there are several publications supporting immune-induced upregulation of the ISGylation machinery, different to its baseline expression levels, and our cells were also stimulated with poly:IC. We also note that multiple epithelial cell lines already express the E3 ligase, HERC5, and multiple studies support that E1/2 are the major rate-limiting steps (Uhlen et al. 2017; Sun et al. 2024; Schneegans et al. 2024) (PMIDs 28818916, 39199018, 38605423). The new Supplementary Figure 21B and its legend are:



Legend: (B) Presence of the full ISGylation machinery (E1/E2/E3 ligases encoded by UBE1L/UBE2L6/HERC5) in HEK293-subclone-ΔISG15 cells. While most HEK293 cells are not known to express E1 (UBE1L) and E2 (UBE2L6) ligases, the western blots show the presence of E1/E2/E3 ligases in our HEK293-subclone-ΔISG15 cells (first lane, labeled C). Furthermore, upregulation of the ISGylation machinery is seen after poly:IC or VSV treatment (as indicated), or with 50 ng of E1 (UBE1L) or E2 ligase (UBE2L6) overexpression in the HEK293-subclone-ΔISG15 cells. Hela cells (last lane) are deficient in the ISGylation machinery (Uhlen et al. 2017) and serve as a negative control. HERC5 is commonly expressed in a range of cell types with multiple publications showing that only transfection of E1/E2 is sufficient (Uhlen et al. 2017; Sun et al. 2024; Schneegans et al. 2024), so no induction is required. Note that HERC5 levels in Hela cells are also low, but can be seen at higher exposures.

These experiments demonstrate that our HEK293 subclone cells express the entire ISGylation machinery, as the precondition to evaluate ISG15-mediated antiviral differences. To make this important point clear in our revised manuscript, we added to the Figure 4b legend: "The HEK293 subclone was selected for high viral titres, and exhibits altered expression profiles of ISGs/IRFs and expresses the full ISGylation machinery (Supplementary Figure 21)."
Additionally, we changed the wording throughout to clarify that we used a specific HEK293-subclone cell line, combined with ISG15 KO, e.g.. "To experimentally confirm this, we transiently transfected HEK293-subclone-ΔISG15 cells …".

With respect to the size and specificity of the ISGylation bands, we apologize that it was not obvious from the exposure of the previous suboptimal Western blot image that ISGylation bands outside of the 17-35 kDa range are indeed present. Without showing both high and low contrast images, this may have led readers to assume other bands were simply dimers. To correct this, we have added additional representative images from the same sets that are quantified, showing ISGylation bands up to 250 kDa. These bands are well beyond the size of dimers and are highly-specific to the myc-ISG15-expressing lanes (indicated by arrows). These images are also consistent with other published data for ISGylation. These are included in a new panel in Extended Data Figure 9e:



Legend: **e,** HEK293-subclone-ΔISG15 cells were transfected with ISG15 constructs as indicated and with/without NSP3C/L (SARS-CoV-2 PLpro). As per panel c, arrows indicate ISGylation bands, or ISG15 dimer/monomer.

We note that ISGylation of human ISG15 may not appear as strong as for *R. affinis*; however, it is clearly visible in the high contrast image that this seems related to the total ISG15 level in the cells. Furthermore, PLpro clearly reduced ISGylation. We also note that HCoV-229E infection further increased ISGylation, particularly for *R. affinis* ISG15 (likely due to immune induction combined with resistance of its ISG15 to PLpro-mediated cleavage) (shown in Extended Data Figure 9c).

In the previous Supplementary Figure 39 lane 6 (human ISG15 ΔC78, overexpressing PLpro) had more protein than lane 5 (no PLpro overexpression), which is visible as the free ISG15 band was also much more prominent (also visible by more ISG15 monomer in the cell lysate and more secreted ISG15 ΔC78), and this misleadingly indicated that the smear in lane 6 was enhanced. While we agree that this was therefore confusing, we would like to emphasize that the quantifications we provide in Figure 4c,h and Extended Data Figure 9a are all normalized relative to the total ISG15 protein abundance in each sample.

These additional experiments, controls and improved images together with an improved explanation in the results and methods section provide a solid foundation for our experiments and their interpretation, and corroborate our conclusions.

Referee #4 (Remarks to the Author):

In the revised version, the figures and plots supporting the AlphaFold modeling and molecular dynamics better illustrate the confidence in the models and the impact of equilibration length and salt concentration on the simulations. The confusing I-TASSER model and DynaMut analysis have been removed from the manuscript. While AlphaFold models of different dimers are presented, they are deemed unreliable as their confidence levels do not align with the experimental data. A noted contradiction is that the human dimer, expected to be stable, has very poor interface scores. Conversely, the R. sinicus dimer shows generally good scores despite expected instability. The authors' interpretations seem plausible, as the local scores at the interface of the bat model are lower than in other parts of the structure, and it remains unclear whether AlphaFold scores correlate with stability. The molecular dynamics simulation presentation has improved, displaying data on equilibration and salt concentration, following my suggestions.

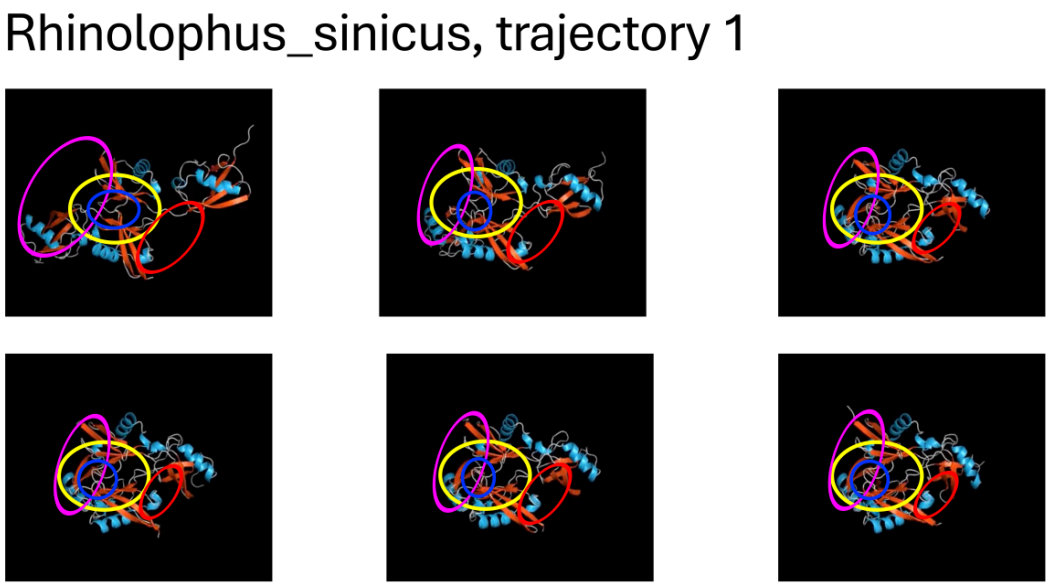
> We thank the reviewer for the helpful comments in the previous and the current revision, as they really improved the presentation of the structural modeling results in the manuscript. We also thank the reviewer for sending snapshots of the dimer model trajectories, which helped us to better understand the discrepancies (addressed below).
> We have now substantiated our results that non-covalent interactions (not only hydrogen bonds but also other interactions) cannot stabilize ISG15 dimers that lack Cys78 and improved the presentation of the ISG15:PLpro models.

However, in my opinion, the molecular dynamics simulation still does not convincingly show that bat ISG15 is unstable:

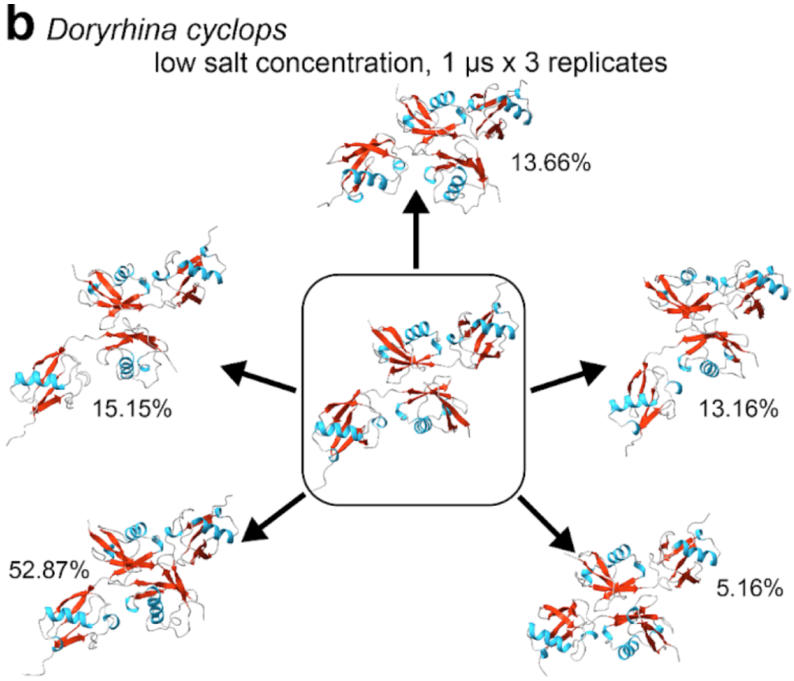
- The two subunits of the bat ISG15 appear to remain attached throughout the simulation, as evidenced by the improved Figures 28 and 29 and the supplementary videos. The interaction interface also seems to be preserved throughout the simulation trajectories. In the review attachment, I provided snapshots from the trajectories for both the human and R. sinicus dimers, which demonstrate the stability of the interfaces for the human dimer (beyond the disulfide bond) but also of the bat dimer interface. The interface, encircled in yellow in the attachment, appears stable and maintains a similar conformation across all snapshots and all three trajectories, or at least is not less stable than in the human simulations. In my opinion, the interface is stable in the molecular dynamics simulation based on this data. This aspect was overlooked in the quantitative evaluation below, likely because the evaluation focused only on hydrogen bonds, as discussed below.

> We thank the Reviewer for their constructive comments. We did not mean to claim that the two monomers of the bat ISG15 remain stably connected throughout the simulations. Rather, we find that they stay near each other but adopt different conformations during the simulation time. Hence, the dynamics of the two monomers of bat ISG15 are not in line with what we would expect if they formed a stable dimer.

Our analyses and videos show that, over the course of the simulations, dimers spend considerable amounts of time in distinct local energy minima. In other words, while we do not observe the monomers disconnecting completely from each other given the available simulation time, we observe a series of alternative structural configurations that (i) differ remarkably among the three replicate simulations and, (ii) importantly differ from the initial AlphaFold structures. This can be seen in the snapshots kindly provided by the reviewer. As one example, we highlight in the *R. sinicus* trajectory 1 image with purple, blue and red circles substantial differences in the monomer arrangements:

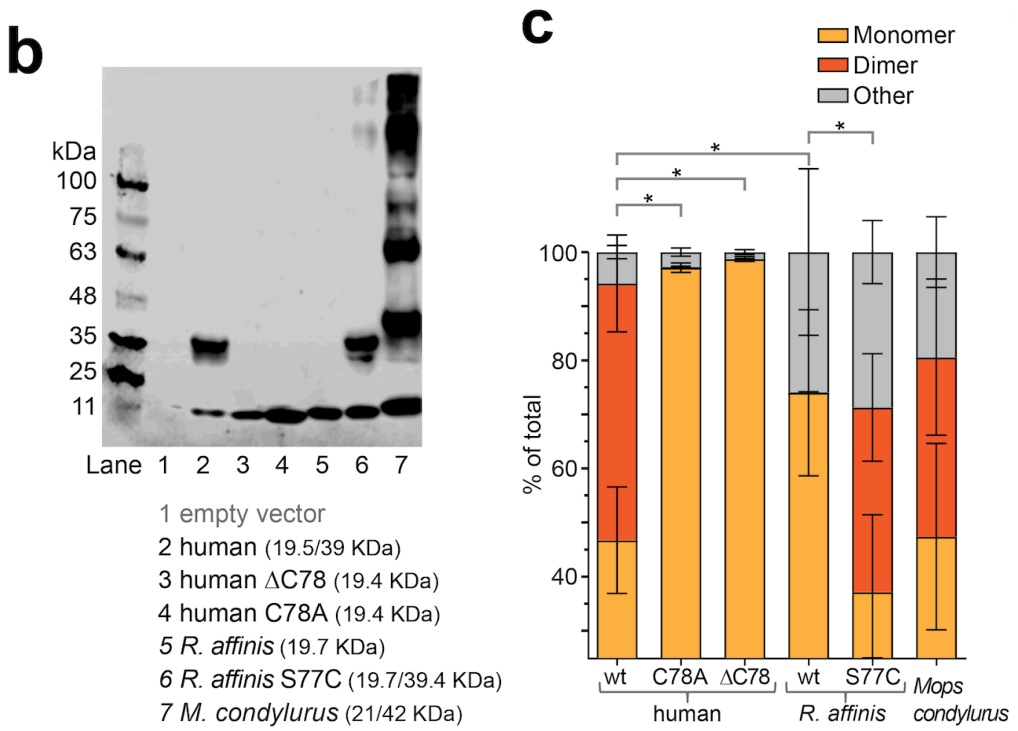


In the manuscript, we illustrate by simulation snapshots presented in the Extended Figure 5, which shows a range of conformations with noticeable differences in the arrangement of the monomers (exemplified in panel b, copied here).



We expect that the two monomers per ISG15 would dissociate fully if the simulation time was greatly increased.

We would also like to emphasize that the simulation results are fully consistent with our laboratory experiments that found no evidence of stable dimer formation for the bat ISG15 or human ISG15 with a mutated Cys78, as shown in Figure 4b,c (copied below).



We address the comment regarding our evaluation of dimer stability based on the contacts formed between monomers below.

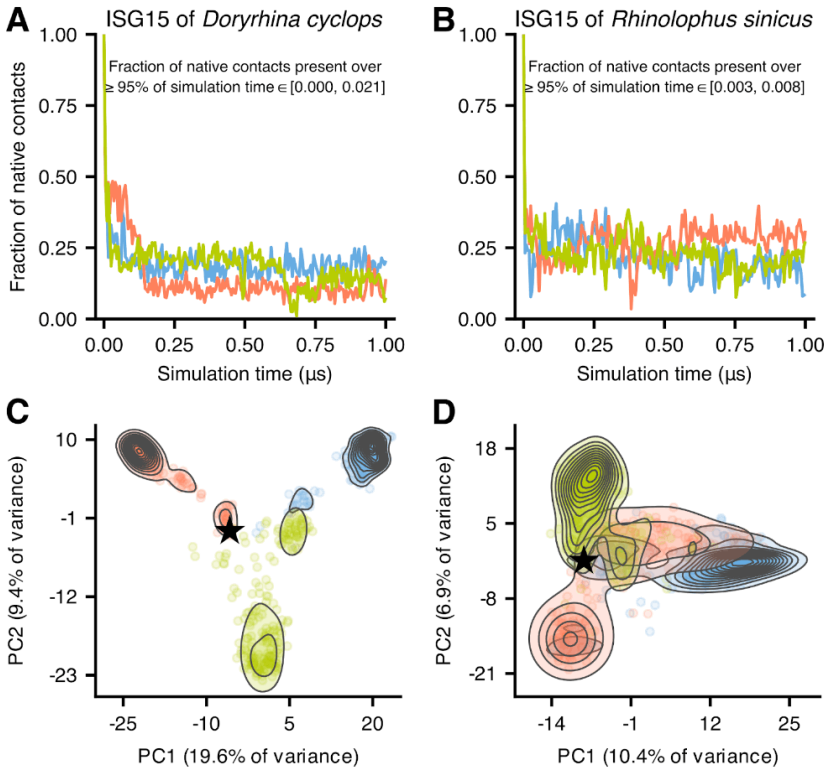
- To provide a quantitative assessment of dimer stability in the simulations, the authors stated: “Similar to the procedure described in (Jandova et al. 2021), we analyzed the fraction of native hydrogen bonds between the two monomers over the course of the simulation.” However, Jandova et al. did not primarily use the fraction of hydrogen bonds, but the fraction of native contacts, calculated with the “gmx hbond-contact” tool, where I believe they meant the gmx hbond program with the -contact option, which focuses on contacts within the cutoff distance rather than hydrogen bonds. Jandova et a. define the fraction of native contacts as “Fnat is the fraction of native contacts that takes into account any atom pair-forming contacts between proteins within a 5 Å distance cutoff”, following the standard in the field. Jandova et al. even noted that “the number of hydrogen bonds shows the smallest importance” in their machine learning classifier of native and non-native interfaces.

The reviewer is correct that the “gmx hbond-contact” tool is gmx hbond with the -contact option. We have corrected this in the methods:
“For the two bat ISG15s, we additionally used the gmx hbond program with the -contact parameter to examine the contacts among ISG15 monomers (within 5Å) that were present at each simulation snapshot. This allowed us to investigate how the fraction of native contacts (i.e., contacts in the original AlphaFold models that are hence present at the beginning of the simulations) changed across simulation time (Supplementary Figure 19).”

- The authors of this manuscript observed very few hydrogen bonds at the interface of the bat dimer but nevertheless then used the fraction of native hydrogen bonds to monitor the stability of the dimer. If the interaction in the model is not mediated by hydrogen bonds, what is the point of using this as a criterion for monitoring stability? Perhaps the interface is more mediated by hydrophobic interactions instead? Monitoring the fraction of native contacts would be more appropriate and independent of the interaction type. Such analysis is necessary minimum to show whether the bat dimers are less stable that the human dimer.

We thank the reviewer for raising the points in these two comments. While we were unaware that the gmx hbond program can also identify contacts other than hydrogen bonds, we have now replaced our hydrogen bond analysis of the bat ISG15 simulations with an analysis of native contacts (the legend in Extended Figure 5 now states “non-covalent interactions (e.g., hydrogen bonds, hydrophobic interactions)”), as suggested by the Reviewer. We found that the fraction of native contacts (contacts present in the initial AlphaFold models) decreases to very low values almost immediately (presented in a new Supplementary Figure 19A that replaces the previous Supplementary Figure 31). The three replicate simulations per ISG15 do not appear to converge to a common fraction value, with the fraction changing within each simulation in a non-stochastic manner. This pattern supports the existence of numerous local energy minima in which simulations get trapped for various periods of simulation time. Importantly, we also note that the fraction of native contacts that are present in at least 95% of simulation time is very low for both bat ISG15’s. This indicates that different native contacts break and re-form during the simulation, and is consistent with native contacts being unable to stabilize dimer formation.

To further substantiate the updated results of native contacts, we have now additionally examined all contacts formed between the two monomers of bat ISG15’s during the simulations. By applying a PCA to the presence/absence matrix of all observed contacts, we found that the three replicate simulations ultimately explore very different areas of the contact space (shown in blue, green and red colors in Supplementary Figure 19B below), far from the initial AlphaFold models. This result is not consistent with the existence of stable ISG15 dimers.



Supplementary Figure 19. Contacts between the ISG15 monomers of *Doryrhina cyclops* (A,C) and *Rhinolophus sinicus* (B,D) observed during the molecular dynamics simulations.

(A,B) Fraction of native contacts (those present in the AlphaFold models) between the two ISG15 monomers. The three colors represent the three replicate simulations. For both species, the fraction of native contacts decreases rapidly in the beginning of the simulations but does not reach zero because the simulations get trapped in different local energy minima for various time periods. Nevertheless, while different native contacts break and re-form during the simulation time, the fraction of native contacts that are maintained in at least 95% of simulation time is not higher than 0.021 and 0.008 for *Doryrhina cyclops* and *Rhinolophus sinicus*, respectively.

(C,D) First two principal components of PCAs of the presence/absence matrices of all contacts (not only native ones) between the two monomers, observed during the simulations. Contour lines denote areas of high density of data points, whereas the stars represent the origin of the three replicate simulations. The contacts formed during each simulation ultimately differ remarkably from those of the AlphaFold models and from each other. This pattern further supports the inability of ISG15 of the two bats to form stable dimers.

We thank the reviewer for these suggestions and believe that the additional analyses have further strengthened our conclusions, which are supported by experimental data.

For the models of PLpro with ISG15, which were added in the revision, only fragmentary data is provided, that does not allow assessing whether the models are good enough for the conclusions about the interfaces:

We apologize for omitting these details and for the inconsistencies in the coloring schemes, which we now all corrected. We also added snapshot figures, as suggested. We thank the reviewer for raising these points.

- No pLDDT or iPTM scores are provided.

We added pLDDT scores and number of contact sites to the legend of the (now) Extended Data Figure 10b and added the color scaling to the figure.

- The PAE plots in Supplementary Figure 41 use a different coloring scheme than Supplementary Figure 24, and the color legend is not provided.

We have modified the PAE plots (now Extended Data Figure 10) to use the same coloring scheme as in Supplementary Figure 16 (Alphafold2 palette) and show the color legend to the right of each plot.

- The legend of Supplementary Figure 41 does not clarify what “differential paired alignments” means. I initially thought the authors meant only regions that differ, but not all regions like those highlighted, so clarification is needed.

We apologize for the misleading terminology. Since the boxes were subject to human interpretation, we have removed this legend phrase and the boxes, and rather show the whole PAE plot.

- There are no figures with the models, just videos, making it very hard to compare the models side by side.

We have corrected this now in Extended Data Figure 10b, where we show snapshots of the model for the whole complex for comparison between the different species, as well as zoomed regions to highlight the contact residues and specifically the C-terminal tail of ISG15 and how it loads into the active site.

- The authors mention “we utilized the recently identified crystal structure” for modeling—does this mean they used the structure as a custom template in ColabFold?

The recently published crystal structure was not a custom template and was in the database for folding.

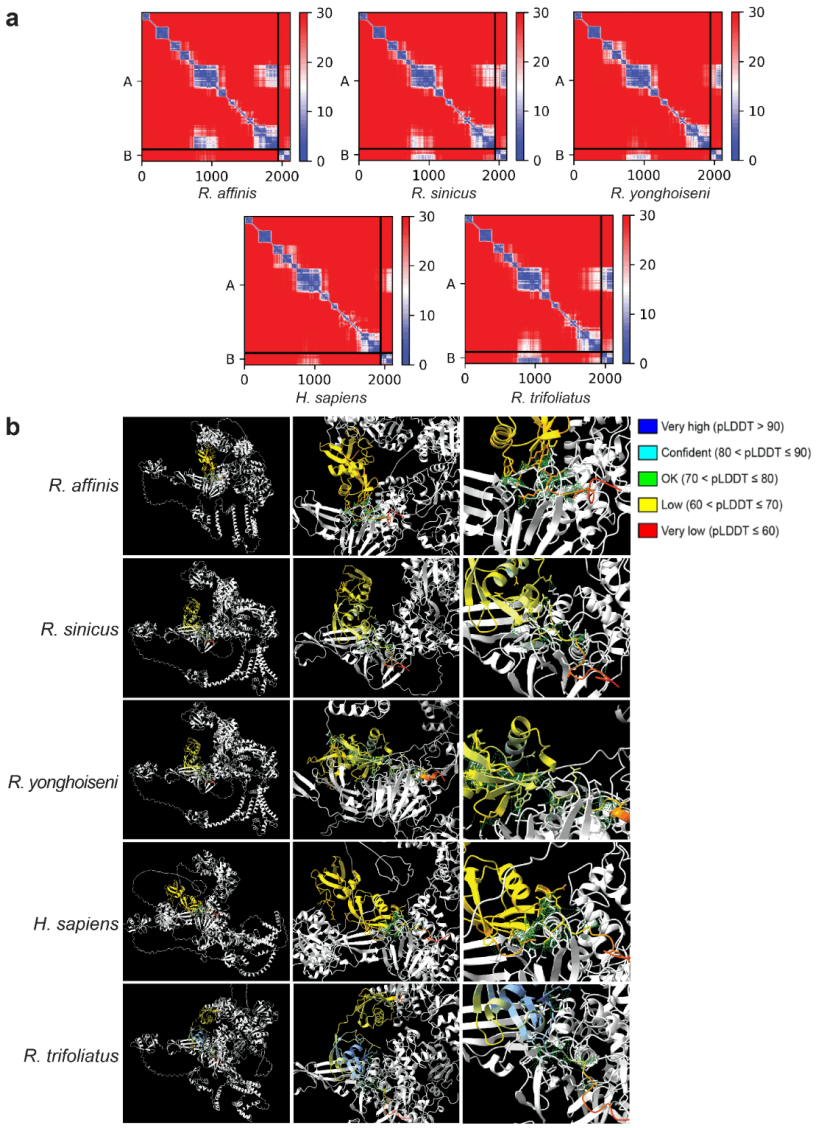
- The authors state: “The human ISG15:PLpro model fits the known structure of the complex well (Supplementary Figure 41)”, but that figure does not contain any comparison to the known structure, just PAE plots—a figure comparing the structure and the model should be included.

We rephrased this sentence to “Compared to the human ISG15:PLpro model, we identified subtle changes in bat ISG15 …”, also to address the next comment below.

- The interfaces discussed on pages 15-16 should be shown in a figure, along with pLDDT coloring, and related to the PAE plots. If the interfaces are not similar to the human structure and exhibit low pLDDT, they should be approached with caution and mentioned as such in the text—in my experience, it is possible to obtain false positive AlphaFold models of interactions, and they often have good PAE but poorer interface pLDDT (as also suggested by the authors for the R.sinicus ISG15 dimer).

The pTM/iPTM scores are rather low, and the human complex (with a well-validated interaction and a published crystal structure) has the lowest score of 0.331/0.32. Several reasons likely contribute to this. First, there are several warnings in the AlphaFold2 documentation about how the pTM may be biased by large size differences between the two multimers, as is the case here. Second, the original crystal structure is a more rigid hexameric form with six monomers of ISG15 interacting with a hexamer of the >2000aa PLpro chain. Hence, a direct comparison between a monomer:monomer model to a hexamer:hexamer crystal structure is difficult, but unfortunately, computational capacity does not allow modeling of the entire hexamer:hexamer complex. Additionally, the reduced database size of the ColabFold online version has been known to falsely decrease pTM confidence of AFM complex structures, even crystal-validated complexes, particularly when there is a di-sulfide bond involved (DOI: 10.1111/tpj.16969). This may also indicate subtle differences between the whole PLpro hexameric complex and Alphafold models of just the 2000 amino acid chain.
Conversely, the iPTM scores for *yonghoiseni*, *sinicus* and *trifoliatus* are all >0.7, indicating higher confidence with the bat ISG15-PLPro molecule interactions.
Nevertheless, our focus here is not on the accuracy of the model prediction, as our experiments already show strong PLpro-mediated ISG15 cleavage and dependency on the LRAA conjugation motif. Instead, we want to highlight that there are subtle species-specific differences between how ISG15 with its conserved C-terminal region loads into PLpro (the unchanging variable).
As requested by the reviewer, we now provide images of the interfaces, showing both zoom views of the active site and the flexible C-terminal ISG15 tail adjacent to the PLpro binding groove with visualized contacts, to Extended Data Figure 10b. Extended Data Figure 10b and the legend also provide pLDDT values and the number of contact sites.

The new Extended Data Figure 10 after including these changes is:



Extended Data Figure 10. Models of SARS-CoV-2 PLpro in complexes with ISG15 of bats and human.

a, Predicted Aligned Error (PAE) plots obtained with Alphafold2-Multimer in ColabFold[17]. Alphafold2 modeled SARS-CoV-2 (wildtype, Wuhan-1) PLpro chain (amino acids 819-2763 of ORF1ab) monomer in complex with a monomer of ISG15 of the five indicated species. The PAE plots represent the error in the position of each amino acid. The crystal structure of PLpro in complex with ISG15 revealed it exists as a pentamer of five units of PLpro with five monomeric ISG15 units in a large complex[18]. This AlphaFold2 model represents the native monomer:monomer structure as folded in Alphafold2-Multimer (Mmseqs2 v 1.5.5) via ColabFold, due to computational limits. See also Supplementary Movies 10-19.

b, Snapshots from Supplementary Movies 10-19 showing PLpro in a complex with ISG15 of bats and human. Images on the left show a whole protein view of the complex (as per panel a). The middle images show a zoom view of the active site and the right images display the flexible C-terminal tail of ISG15 adjacent to the PLpro binding groove with contacts visualized in green (Supplementary Movies 10-19 show the details). pLDDT scores and number of contacts for each of the five species are 64.84: 422, 65.77: 156, 66.04: 761, 60.44: 354, 65.79: 126, respectively. ISG15 is colored by pLDDT values. PLpro in white. The linear C-terminal tail of human ISG15 resembles that of *R. trifoliatus* ISG15, loading directly into the PLpro catalytic site, with additional contact sites in *R. trifoliatus* that seem to stabilize the interaction.