nature portfolio

Peer Review File

Bat-specific adaptations in interferon signaling and GBP1 contribute to enhanced antiviral capacity

Corresponding Author: Dr Arinjay Banerjee

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This article characterizes antiviral pathways induced by INF beta signaling in two bat species, Pteropus alecto and Eptesicus fuscus, and compare them to the equivalent response in human cell lines. Authors found species-specific roles of STAT1 and STAT2 in INF-beta signaling and using transcriptomic approach they identified non-canonical interferon-stimulated bat gene GBP1, with the enhanced antiviral activity against several RNA and DNA viruses. The work provides novel insights into the evolution of interferon-mediated antiviral responses in bats.
However, different concerns should be addressed to improve the quality of the current manuscript.

1. Authors claim the role of adaptation in interferon signaling and GBP1 in the enhanced viral tolerance in bats. How do they distinguish between viral tolerance and control of viral infection (or viral resistance)? The presented results favor control of viral infection rather than tolerance to it. These concepts should be clarified.

2. Authors generated recombinant IFN beta specific for P. alecto and E. fuscus species, and compared their activity to human IFN beta. Could they present in supplementary data the sequences of generated IFN beta? Does their vehicle control GFP contain secreted GFP?

3. Only immortalized bat and human cell lines were used in study (human RPTEC-hTERT line is not primary cell line, as indicated in methods). As immortalization could change different aspects of cell biology it is important to validate obtained results in primary bat and human cells.

4. How do authors explain the high difference between human A549 and RPTEC cells in the dose of IFN beta necessary to protect against VSV infection, shown at figure S1B?

5. Nuclear translocation of STAT1 and 2 is not clear in figures S2 and S6. Could it be quantified?

6. In the legend of figure 2D it needs to be indicated what experimental conditions does “DKD” present?

7. Line 63: as the antiviral function of GBP1 was first described in 1999, it could not be presented as “recently discovered ISG”.

8. Results presented in the Fig. 3G do not favor an important effect of GBP1 in the control of H1N1 infection. Par consequence, the discussion, lines 736-740, will need to be modified.

9. The important part of the study, role of bat GBP1 mutation, was tested only in human A549-ACE2 cell line. Could that effect be reproduced in some primary cell line?

10. Authors have analyzed mainly interferon type I signaling but they use the term bat “interferome” throughout the manuscript, which implicates interferon type I, II and III signaling-regulated genes. This is not appropriate in most of the manuscript and will need to be modified or justified.

Reviewer #3

(Remarks to the Author)
In this work, the authors have carried out a extensive range of virological and molecular assays to study the bat interferon response in two bat species, including focused analyses of several ISGs and the STAT signal transduction pathway.
I find the manuscript diverse, with an impressive array of experiments with different viruses and with many novel and important findings. I have a few concerns as well as some technical suggestions I list below. Furthermore, some of the terminology used by the authors should be rephrased or clarified.

My major comment is that the use of cell lines from different species to compare the innate immune response between these species obviously entails certain biases and this should be acknowledged in the beginning of the manuscript. Further details regarding the particular choice of these cell lines (how similar they are in terms of tissue and lineage) as well as how they were immortalized and what is known about how this immortalization may have impacted gene expression (especially with regards to the IFN pathway) each cell line should be given. Finally, when cross-species comparisons are based on the use of these cells, a cautionary note should be added (e.g., when comparing basal or induced expression levels, when discussing kinetics of response – all of these can be heavily biased by the use of these particular cell lines).

I find the bat GBP1-specific activity against the bat poxvirus to be the most novel and unique result. However, it is somehow "missed", appearing towards the end of the manuscript. I recommend the authors to more strongly and clearly emphasize this important results earlier in the manuscript. In this way, it will not be lost by future readers.

Additional comments:

In Abstract:
"Using transcriptomic analysis, we identified canonical and non-canonical interferon stimulated genes including two key bat genes, IFIT1 and GBP1.." the term "key bat genes" is inaccurate, please rephrase.

In Introduction: The section between lines 51-62 should have more citations regarding the various details given on innate immune response.

In Methods:

In "herpes simplex virus 1 encoding GFP (HSV1-GFP)" – more details should be given regarding this virus: where is the GFP in the viral genome when is it expressed during replication and where in the cell?

Lines 189-190: "RNA Sequencing: PaKiT03, Efk3B, A549, and RPTEC cells were untreated or treated with 1U/mL of species matched IFNβ for 6 hours." How many replicates per cell per condition were used?

Transcriptome used for mapping: For clarity & reproducibility, more details should be given:
e.g., in - GCF_000325575.1 – from NCBI, there can be several sets used: which sets of genes did you use? Refseq? GenBank?

The orthology procedure is not clear and is not sufficiently detailed:
Lines 205-207:
"OrthoFinder v2.5.2 was applied pair-wise across the three species, with each bat gene set being mapped back to human genes, and any remaining unmapped genes in an orthogroup between the two bat species also being mapped together"
Following OrthoFinder run, how did you define orthologs? Did you take all orthogroups? Did you use only one-to-one in the following analyses? If not, how did you treat cases of one-to-many or many-to-many orthologs?

Antiviral Efficacy of GBP1: Add details on regarding why the MOI used (0.01) chosen?
In the case of MERS-CoV (line 219), I guess there is a missing number – the current version has "MOI 0.0"

"A549-ACE2 cells were transfected with 250-1000ng of each construct using 218 Lipofectamine 3000" – How many cells were plated and in what dish size were they plated?

In Results:

Fig 3B:
It is not clear which conditions are included in the PCA – are these only treated or only non-treated samples or both? If it includes both conditions for each species, did you test whether other PCs (PC3,4) show separation by treatment? (or if all treated are grouped together with respect to untreated, within each species' cluster?)
It is also unclear what is meant by "There were limited perturbations between the kidney cell lines along principal component one (PC1), which accounts for more than 67% of sample variation."
Do you mean that the samples are not separated by condition in PC1/2?

Fig 3C is a rather crude analysis: you use a cutoff to determine whether a given GO term is enriched or not in any cell line (and if a given term is slightly below the cutoff it will be called as non-significant), and each of these processes is based on a list of genes annotated as belonging to it (and this is an inaccurate annotation with false negatives and positives). I would

either remove this panel altogether or instead discuss specific genes uniquely upregulated instead (e.g., the ones you already mention - OAS3, PARP9, IFIH1 and ADAR). The analysis in Fig 3D is more careful and appropriate for such data analysis as GO terms.

Fig 3F:
It is unclear what is meant by "top 27 bat DEGs" – in both Fig3F and Supp Fig9, it seems that these genes are highly upregulated in human cells and the upregulation in bat cells is milder – are these the 27 genes that have the highest fold change in response in the bat cells? If so, is it in both of them? One of them? What was the metrics of this definition? Additionally, the genes that appear in multiple copies in bats should be called "paralogs" and not "variants". To avoid confusion in the heatmaps themselves, I would also either merge the human rows that represent the same gene (E.g., in the BST2 variants into one) or just show the first row and leave the rest as grey / white, to emphasize the fact there is no more than a single human gene.

Supp Fig 9C-D & related text in main text (lines 477-487): The text mentions several genes in a confusing manner – they are described as "bat specific DEGs that we could not directly map back to the human genome annotation", but some of these genes should have clear one-to-one orthologs in the human genome (DDX58, OAS1,LGALS9), while others have been lost in one bat species (IFI44, IFI44L – as can been seen in the matrix). These should not be grouped together and different terms should describe them. Additionally, the fact that Megabats, but not Vespertilionidae, have lost the IFI44/IFI44L locus and that these genes are highly upregulated in innate immune response of bats species that did not lose them was mentioned by Schneor et al (PMID: 37575178), and can be referenced as it is an analogous finding in different species and cells that nicely fits the data presented here.

With respect to: "Bat specific DEGs also include several unannotated and uncharacterized genes such as LOC102882974, LOC112476441, LOC129151230, and LOC129150681" – have you tried to BLAST them against all mammals to see if homology is detected to a functionally known gene? The fact they lack names and appear as "LOC" does not always mean that their likely function cannot be inferred but rather that they may belong to a multi-copy gene family or encode for endogenous retroelements.

With respect to: "In P. alecto bats, we identified one IFIT1 gene (LOC102878285) that mapped back to the human genome (Supplementary Figure 11)." Instead, or in addition, to the sequence alignment, I would suggest adding a tree that is based on the sequences of all IFIT paralogs and orthologs in human, mouse and these two bat species, to show which genes cluster together. The statement of "mapped back to" is not clear and should be supported by a tree-based analysis or another analysis that shows these genes have greatest similarity to this particular human paralog.

The motifs mentioned in GBP1 and IFIT1: it should be clarified whether these motifs are "classical" linear motifs found in disordered regions (and possibly also annotated in databases such as the ELM database).

In Discussion:

In lines 632-633:
"These findings suggest an evolutionary adaption of the type I IFN response in bat species, which in turn may lead to a species-specific antiviral response in bats."
The mentioned findings do not necessarily suggest adaptation and/or species-specific antiviral response in bats. Rather, they suggest that there is rapid coding sequence evolution of Type I IFNs and their receptors, such that IFNs from various species cannot bind efficiently IFNARs and elicit a strong response. Universal IFN is an artificial construct that (rather weirdly) works on several species' IFNAR, but it is not directly related to species-adaptation in these genes.

The following sentence (lines 675-676):
"This selection has been shown to be even higher in bats in comparison to other mammals" is phrased in an unclear manner – what do you mean by "even higher in bats"? Please clarify or rephrase.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Authors have successfully answered to all the questions.

Reviewer #3

(Remarks to the Author)
The authors have addressed my questions & concerns and I have no further questions.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This article characterizes antiviral pathways induced by INF beta signaling in two bat species, Pteropus alecto and Eptesicus fuscus, and compare them to the equivalent response in human cell lines. Authors found species-specific roles of STAT1 and STAT2 in INF-beta signaling and using transcriptomic approach they identified non-canonical interferon-stimulated bat gene GBP1, with the enhanced antiviral activity against several RNA and DNA viruses. The work provides novel insights into the evolution of interferon-mediated antiviral responses in bats.
However, different concerns should be addressed to improve the quality of the current manuscript.
Authors' response: We thank the reviewer for appreciating our study and for noting the novel insights that our study provides into the evolution of interferon-mediated antiviral responses in bats. We have addressed this reviewer's concerns below.

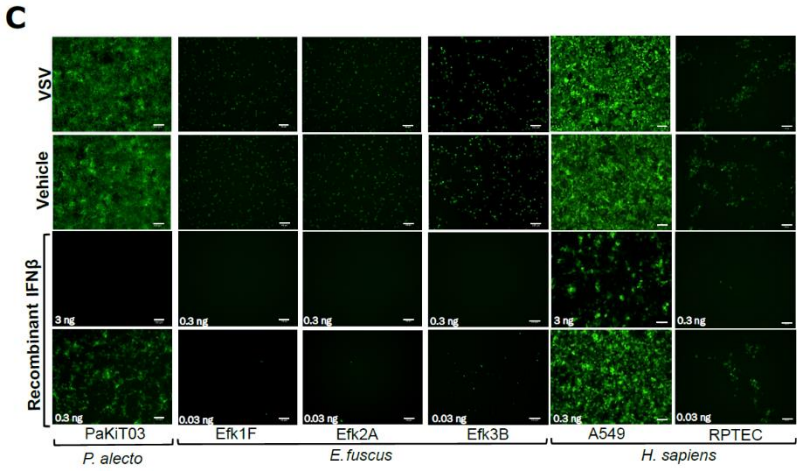
1. Authors claim the role of adaptation in interferon signaling and GBP1 in the enhanced viral tolerance in bats. How do they distinguish between viral tolerance and control of viral infection (or viral resistance)? The presented results favor control of viral infection rather than tolerance to it. These concepts should be clarified.
Authors' response: Thank you for the comment. We have rephrased the abstract to include: *Here, we characterized antiviral pathways in two divergent bat species, Pteropus alecto and Eptesicus fuscus, identifying unique bat-specific mechanisms underlying their enhanced control of viral infection.* In addition, any text where we stated "viral tolerance" has been modified to "*antiviral capacity"*

2. Authors generated recombinant IFN beta specific for P. alecto and E. fuscus species, and compared their activity to human IFN beta. Could they present in supplementary data the sequences of generated IFN beta? Does their vehicle control GFP contain secreted GFP?
Authors' response: Thank you for the suggestion. We have now included in Supplementary Figure 1 an alignment of the three IFNβ sequences. We have also now explicitly mentioned in the text that the vehicle control is secreted GFP. We have also added a sentence in the methods section: *A vehicle control, supernatant containing secreted GFP, was also generated following this procedure.*

3. Only immortalized bat and human cell lines were used in study (human RPTEC-hTERT line is not primary cell line, as indicated in methods). As immortalization could change different aspects of cell biology it is important to validate obtained results in primary bat and human cells.
Authors' response: We thank the reviewer for this comment. RPTEC-hTERT is a life-extended human cell line. We have clarified this in the methods.

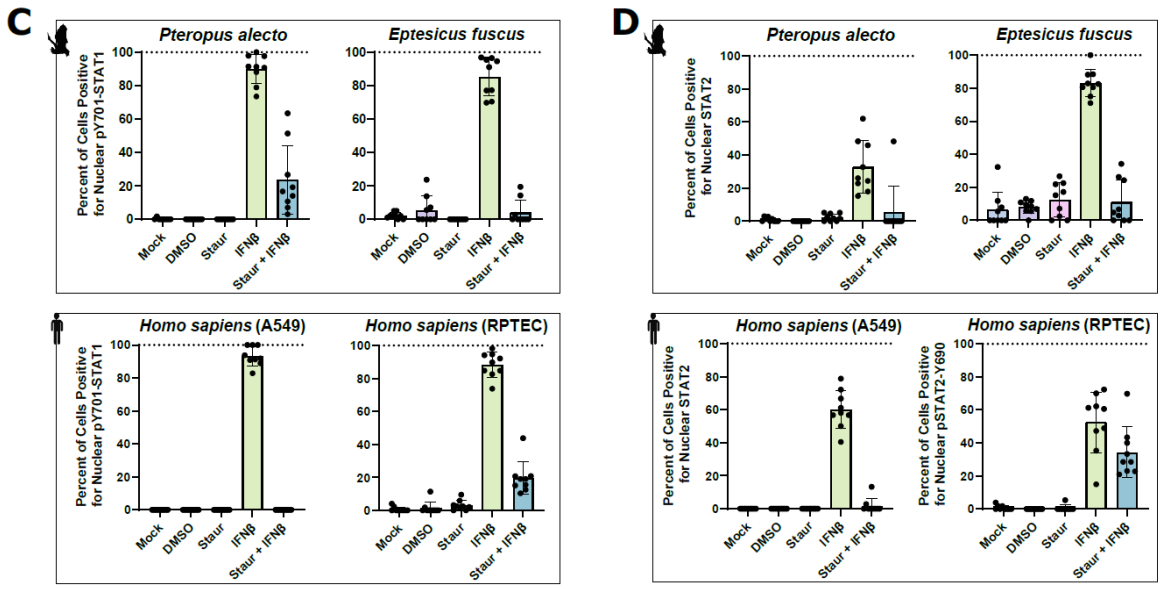
As pointed out by the reviewer, to demonstrate that IFNβ-mediated responses are not significantly impacted by the immortalization process, we have now tested IFNβ-mediated protection against VSV-GFP in multiple clones of *E. fuscus* cells, like Efk-1F and Efk-2A. Since primary cells from this bat species are not available, we believe that demonstrating that clonal populations of immortalized *E. fuscus* cells respond to IFNβ treatment highlights that

immortalization process of these cells did not significantly impact IFN responses. We have now also included these additional data in Supplementary Figure 1C, where 0.3ng protects all Efk clonal cells. The manuscript has been modified to read: *We also validated IFNβ activity in multiple clonal populations of E.fuscus cell lines, such as Efk1F and Efk2A (**Supplementary Figure 1C**)[8,9]. Equivalent amounts of E. fuscus IFNβ were required for antiviral protection against VSV-GFP in all three clonal cell lines.*



4. How do authors explain the high difference between human A549 and RPTEC cells in the dose of IFN beta necessary to protect against VSV infection, shown at figure S1B?
Authors' response: IFNβ-mediated protection depends on several factors, including the availability, distribution, and concentration of IFNAR1/2 receptors on the surface of the cells, along with differences within the cells, like transcription factors. Indeed, differences observed between A549 and RPTEC cells represents cell-type specific responses since both cell lines are derived from very distinct human tissues, lung and kidney, respectively.

5. Nuclear translocation of STAT1 and 2 is not clear in figures S2 and S6. Could it be quantified?
Authors' response: While the nuclear translocation was quantified for Supplementary Figure 6 and displayed in Figure 2B, this analysis was not performed for Supplementary Figure 2B. We have now done so and have included it as an additional panel in Supplementary Figure 2 and is referred to appropriately within the main text. New supplementary figure 2B is also copied below.



6. In the legend of figure 2D it needs to be indicated what experimental conditions does "DKD" present?
Authors' response: Thank you. We have now modified the figure legend to read: *PaKiT03, Efk3B, A549, and RPTEC cells were pretreated for 48 hours with siRNA targeting STAT1 and STAT2 individually or in combination (DKD).*

7. Line 63: as the antiviral function of GBP1 was first described in 1999, it could not be presented as "recently discovered ISG".
Authors' response: We thank the reviewer for their thoroughness. Since we had to significantly reduce the word count in the revised manuscript, the mention of '*recently discovered ISG'* has now been removed.

8. Results presented in the Fig. 3G do not favor an important effect of GBP1 in the control of H1N1 infection. Par consequence, the discussion, lines 736-740, will need to be modified.
Authors' response: These sentences have now been removed from the revised manuscript as part of reducing the word count of the main text.

9. The important part of the study, role of bat GBP1 mutation, was tested only in human A549-ACE2 cell line. Could that effect be reproduced in some primary cell line?
Authors' response: We thank the reviewer for this comment. As highlighted above, since we validated GBP1 as a ***direct acting*** antiviral gene, the function of the gene should be cell-type independent. We have included additional citations below for direct acting ISGs, including our most recent publication where we characterized bat and human ISG15 in human lung (A549) cells.

Examples of direct acting ISG manuscripts
Our ISG15 article as an example – all bat ISG15s were expressed in A549 cells
- https://www.nature.com/articles/s41586-024-08471-0

The study below studied the effect of porcine GBP1 in both porcine and human (HEK293T) cells. They were able to characterize the effect of porcine GBP1 against classical swine fever virus and identify the residues in GBP1 that are critical for this antiviral activity.

- https://pmc.ncbi.nlm.nih.gov/articles/PMC4836331/

This study used HEK293T cells to validate the interaction of the Chinese tree shew GBP1 with the phosphoprotein of VSV. This interaction reduces VSV genomic transcription, inhibiting virus replication.

- https://www.sciencedirect.com/science/article/pii/S104346662030404X?via%3Dihub#s0010

Another study identified bat ASC2 as a negative regulator of inflammasomes and studied its function in humans and mice. It was found to reduce inflammation and improve outcomes in a mouse model of virus infection.

- https://www.cell.com/action/showPdf?pii=S0092-8674%252823%252900333-1

10. Authors have analyzed mainly interferon type I signaling but they use the term bat "interferome" throughout the manuscript, which implicates interferon type I, II and III signaling-regulated genes. This is not appropriate in most of the manuscript and will need to be modified or justified.

Authors' response: We have now modified the text within the manuscript to include *type I IFN response* and removed the word *interferome*.

Reviewer #3 (Remarks to the Author):

In this work, the authors have carried out a extensive range of virological and molecular assays to study the bat interferon response in two bat species, including focused analyses of several ISGs and the STAT signal transduction pathway.

I find the manuscript diverse, with an impressive array of experiments with different viruses and with many novel and important findings. I have a few concerns as well as some technical suggestions I list below. Furthermore, some of the terminology used by the authors should be rephrased or clarified.

My major comment is that the use of cell lines from different species to compare the innate immune response between these species obviously entails certain biases and this should be acknowledged in the beginning of the manuscript. Further details regarding the particular choice of these cell lines (how similar they are in terms of tissue and lineage) as well as how they were immortalized and what is known about how this immortalization may have impacted gene expression (especially with regards to the IFN pathway) each cell line should be given. Finally, when cross-species comparisons are based on the use of these cells, a cautionary note should be added (e.g., when comparing basal or induced expression levels, when discussing kinetics of response – all of these can be heavily biased by the use of these particular cell lines).

I find the bat GBP1-specific activity against the bat poxvirus to be the most novel and unique result. However, it is somehow "missed", appearing towards the end of the manuscript. I recommend the authors to more strongly and clearly emphasize this important results earlier in the manuscript. In this way, it will not be lost by future readers.
Authors' response: We thank the reviewer for appreciating our study and for highlighting our novel and important findings.

We have acknowledged within the discussion section that our study provides a framework to study bat IFN signaling and their ISG repertoire. We highlight that although our studies were performed in two bat species using two cell types, additional studies are required to characterize the full breadth of IFN-mediated antiviral responses in bat species using diverse cell types derived from different tissues and from different bat species. We have added the following to the discussion section of the main text: *Although our studies were performed in two bat species using two well characterized cell types, additional studies are required to characterize the full breadth of IFN-mediated antiviral responses using diverse cell types derived from different tissues and from different bat species, along with making these tools available to the research community*[55].

We realize that there are very limited bat cells that are commercially available for bat research. Citation 55 in the paragraph above refers to our most recent work where we developed several new cell lines from *Carollia perspicillata* bats. We made some of these cell lines available for research through BEI resources and ATCC (Gonzalez *et al.,* PLOS Biology, 2025).

We have now mentioned the immortalization process for the two bat cell lines in the methods section of the manuscript. Both bat cells lines were immortalized using polyomavirus T antigens. Also, in the methods section, we mention that both bat cells are of kidney origin. The two selected cell lines have been used extensively in bat research for over 15 years and both cell lines have been shown to have an intact antiviral interferon-mediated response, along with proinflammatory responses and the existence of the inflammasome. These cells have been used for performing CRISPR screens and for the generation of CRISPR knockout cell lines.

We thank the reviewer for highlighting the novel and unique activity of bat GBP1 against bat poxvirus (EfPV). We have now mentioned the bat GBP1 activity against EfPV in the abstract and within the last paragraph in the introduction section as well to ensure that this important finding is not missed.

Additional comments:

In Abstract:
"Using transcriptomic analysis, we identified canonical and non-canonical interferon stimulated genes including two key bat genes, IFIT1 and GBP1.." the term "key bat genes" is inaccurate, please rephrase.
Authors' response: We have now replaced 'key bat genes' with '*two differentially expressed genes'.*

In Introduction: The section between lines 51-62 should have more citations regarding the various details given on innate immune response.

Authors' response: Due to the reference number limitation, we could only cite one additional review article which describes the JAK-STAT signaling pathway in humans and how its activation leads to interferon stimulated gene expression (https://www.nature.com/articles/s41392-021-00791-1)

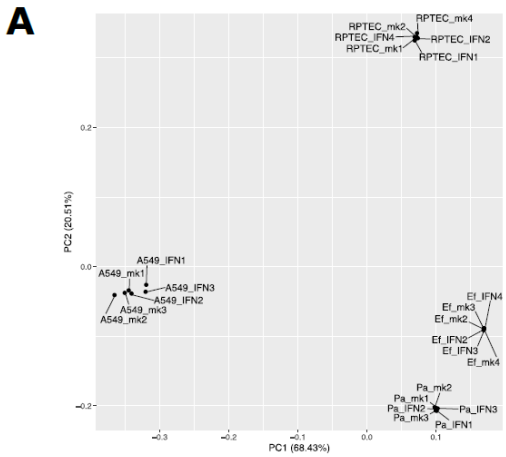
In Methods:

In "herpes simplex virus 1 encoding GFP (HSV1-GFP)" – more details should be given regarding this virus: where is the GFP in the viral genome when is it expressed during replication and where in the cell?
Authors' response: HSV1-GFP was kindly provided by Dr. Karen Mossman (McMaster). In this recombinant virus, GFP is fused to the capsid protein VP26, allowing GFP to be expressed in both the nucleus and the cytoplasm. We have now indicated in the methods that we are using "*HSV1-K26-GFP".*

Lines 189-190: "RNA Sequencing: PaKiT03, Efk3B, A549, and RPTEC cells were untreated or treated with 1U/mL of species matched IFNβ for 6 hours." How many replicates per cell per condition were used?
Authors' response: We have now mentioned the number of replicates (n=3/condition).

Transcriptome used for mapping: For clarity & reproducibility, more details should be given: e.g., in - GCF_000325575.1 – from NCBI, there can be several sets used: which sets of genes did you use? Refseq? GenBank?
Authors' response: Thank you for the comment. With respect to GCF_000325575.1, there is only one RefSeq gene annotation whereas GCA_000325575 is the GenBank annotation. To make this clear, we have included "RefSeq" as follows: *Transcriptome indices based on the RefSeq annotations for Pteropus alecto (GCF_000325575.1), Eptesicus fuscus (GCF_027574615.1), and Homo sapiens (GCF_000001405.40) were created with salmon v1.4.0 using their genomes as a decoy."*

The orthology procedure is not clear and is not sufficiently detailed. Lines 205-207: "OrthoFinder v2.5.2 was applied pair-wise across the three species, with each bat gene set being mapped back to human genes, and any remaining unmapped genes in an orthogroup between the two bat species also being mapped together". Following OrthoFinder run, how did you define orthologs? Did you take all orthogroups? Did you use only one-to-one in the following analyses? If not, how did you treat cases of one-to-many or many-to-many orthologs?
Authors' response: All genes in an orthogroup were defined as each others' ortholog. We have now added this to the text for clarification. The manuscript now reads: *OrthoFinder v2.5.2 was applied pair-wise across the three species, with each bat gene set being mapped back to human genes, and any remaining unmapped genes in an orthogroup between the two bat species also being mapped together. All genes in an orthogroup were defined as each others' ortholog.*



Antiviral Efficacy of GBP1: Add details on regarding why the MOI used (0.01) chosen?
In the case of MERS-CoV (line 219), I guess there is a missing number – the current version has "MOI 0.0"

Authors' response: We have corrected MERS-CoV MOI to 0.1. We have also mentioned in the manuscript that we chose an MOI of 0.1 to assess the antiviral role of GBP1 during multiple rounds of virus replication in culture. Furthermore, we did not want to overwhelm the culture system with too much virus that could potentially bypass GBP1-mediated restriction, although this is an intriguing question that we are now exploring in a subsequent study.

Next text added to manuscript: *A low MOI was chosen to assess the antiviral role of GBP1 during multiple rounds of virus replication in culture and to not overwhelm the culture system with too much virus that could potentially bypass GBP1-mediated restriction. As A549 cells are less permissive to MERS-CoV, a higher MOI was utilized.*

"A549-ACE2 cells were transfected with 250-1000ng of each construct using 218 Lipofectamine 3000" – How many cells were plated and in what dish size were they plated?

Authors' response: We have now added this information in the manuscript within the methods section. Specifically, we have added – *A549-ACE2 cells ($1.5x10^5$ cells/well) seeded in a 12 well plate were transfected with 250-1000ng of each construct using Lipofectamine 3000 (Thermo Scientific) .*

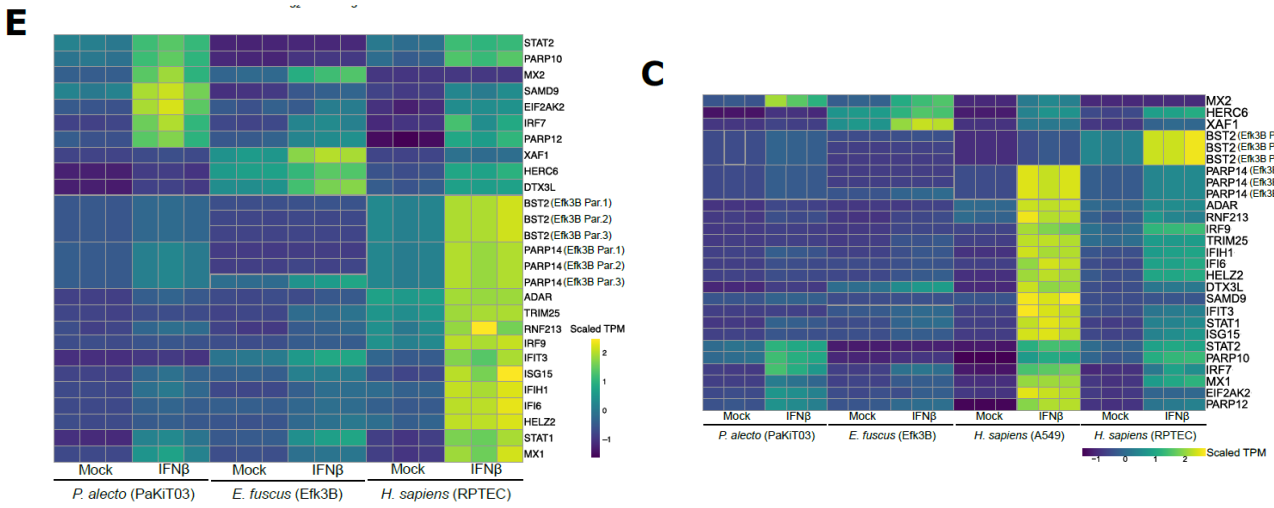
In Results:

Fig 3B:
It is not clear which conditions are included in the PCA – are these only treated or only non-treated samples or both? If it includes both conditions for each species, did you test whether other PCs (PC3,4) show separation by treatment? (or if all treated are grouped together with respect to untreated, within each species' cluster?)
It is also unclear what is meant by "There were limited perturbations between the kidney cell lines along principal component one (PC1), which accounts for more than 67% of sample variation." Do you mean that the samples are not separated by condition in PC1/2?

Authors' response: We didn't look at other PCs. PCAs of individual cell lines showed clear separation between treated and untreated, but when assessing all the cell lines together, the differences between cell lines seems to drive the majority of the variation. We have now modified Supplementary Figure 9 to include the untreated samples in the provided PCA plot (Supplementary Figure 9A).

Fig 3C is a rather crude analysis: you use a cutoff to determine whether a given GO term is enriched or not in any cell line (and if a given term is slightly below the cutoff it will be called as non-significant), and each of these processes is based on a list of genes annotated as belonging to it (and this is an inaccurate annotation with false negatives and positives). I would either remove this panel altogether or instead discuss specific genes uniquely upregulated instead (e.g., the ones you already mention - OAS3, PARP9, IFIH1 and ADAR). The analysis in Fig 3D is more careful and appropriate for such data analysis as GO terms.
Authors' response: We agree with the reviewer's comment and have now removed the Venn Diagram from Figure 3 to focus on the more in-depth analysis performed for panel D.

Fig 3F:
It is unclear what is meant by "top 27 bat DEGs" – in both Fig3F and Supp Fig9, it seems that these genes are highly upregulated in human cells and the upregulation in bat cells is milder – are these the 27 genes that have the highest fold change in response in the bat cells? If so, is it in both of them? One of them? What was the metrics of this definition?
Additionally, the genes that appear in multiple copies in bats should be called "paralogs" and not "variants". To avoid confusion in the heatmaps themselves, I would also either merge the human rows that represent the same gene (E.g., in the BST2 variants into one) or just show the first row and leave the rest as grey / white, to emphasize the fact there is no more than a single human gene.
Authors' response: Thank you for the suggestions. We have clarified the statement made in the Figure 3 legend to read: *Heatmap indicating the expression levels of the top DEGs in bats ranked by p-value involved in the IFNβ response*. These genes were the top ranked in either or both bat specie and are more mildly upregulated compared to the human cells evaluated, leading us to speculate whether these antiviral genes have a higher **functional** potency at lower levels of expression in bats. Our manuscript further supports this as *E. fuscus* GBP1 exhibited antiviral potency at lower levels of expression against Eptesipoxvirus, compared to human GBP1.
In addition, the heatmaps in Figure 3 and Supplementary Figure 9C have been modified to read paralogs and the human rows are merged to demonstrate that the same gene is being represented.



Supp Fig 9C-D & related text in main text (lines 477-487): The text mentions several genes in a confusing manner – they are described as "bat specific DEGs that we could not directly map back to the human genome annotation", but some of these genes should have clear one-to-one orthologs in the human genome (DDX58, OAS1,LGALS9), while others have been lost in one bat species (IFI44, IFI44L – as can been seen in the matrix). These should not be grouped together and different terms should describe them. Additionally, the fact that Megabats, but not Vespertilionidae, have lost the IFI44/IFI44L locus and that these genes are highly upregulated in innate immune response of bats species that did not lose them was mentioned by Schneor et al (PMID: 37575178), and can be referenced as it is an analogous finding in different species and cells that nicely fits the data presented here.

Authors' response: We have now modified the text so that it describes the data more accurately. We have added the following to the results and discussion:

*We also analyzed the expression levels of bat specific DEGs that we could not directly map back to the human genome annotation, such as IFI27-like, TRIM5a-like, OAS1, SP140-like, LOC112483779 (unnamed), DDX58, and DDX60L, (**Supplementary Figures 9D-E and Supplementary Table S7**). Additionally, IFI44 and IFI44L is have been lost in P. alecto (**Supplementary Figures 9D**).*

*While duplications are common in the Vespertilionidae bat family, deletion of genes like IFI44 and IFI44L (**Supplementary Figure 9D**) have been reported for the Pteropodidae bat family[9fff6]. Notably, IFI44L was readily upregulated in E. fuscus cells upon stimulation, demonstrating additional variation in the antiviral response across bats.*

With respect to: "Bat specific DEGs also include several unannotated and uncharacterized genes such as LOC102882974, LOC112476441, LOC129151230, and LOC129150681" – have you tried to BLAST them against all mammals to see if homology is detected to a functionally known gene? The fact they lack names and appear as "LOC" does not always mean that their likely function cannot be inferred but rather that they may belong to a multi-copy gene family or encode for endogenous retroelements.

Authors' response: Yes, we tried to blast all genes that were listed as an LOC. The genes that had a gene symbol (for example, *P. alecto* IFIT1) were modified, with their LOCs identified in Supplementary Table 7. All the LOCs that are listed in Supplementary Figure 9C-D are uncharacterized and undefined, and that is further exemplified in Supplementary Table 7. The information for these uncharacterized/undefined genes as found on NCBI is copied below:

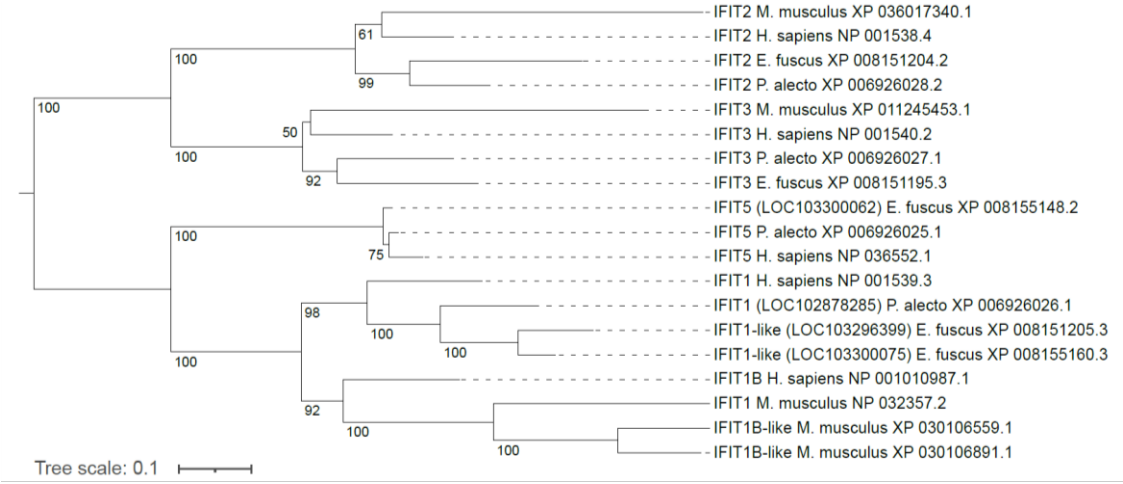
LOC102882974: lncRNA;model_evidence=Supporting evidence includes similarity to: 100%25 coverage of the annotated genomic feature by RNAseq alignments%2C including 24 samples with support for all annotated introns;product=uncharacterized LOC102882974;transcript_id=XR_439218.3

LOC112476441: lncRNA;model_evidence=Supporting evidence includes similarity to: 100%25 coverage of the annotated genomic feature by RNAseq alignments%2C including 6 samples with support for all annotated introns;product=uncharacterized LOC112476441;transcript_id=XR_003045097.1

LOC129151230: lncRNA;ID=gene-LOC129151230;Dbxref=GeneID:129151230;Name=LOC129151230;description=uncharacterized LOC129151230;gbkey=Gene;gene=LOC129151230;gene_biotype=lncRNA

LOC129150681: protein_coding;description=uncharacterized LOC129150681;gbkey=Gene;gene=LOC129150681;gene_biotype=protein_coding

With respect to: "In P. alecto bats, we identified one IFIT1 gene (LOC102878285) that mapped back to the human genome (Supplementary Figure 11)." Instead, or in addition, to the sequence alignment, I would suggest adding a tree that is based on the sequences of all IFIT paralogs and orthologs in human, mouse and these two bat species, to show which genes cluster together. The statement of "mapped back to" is not clear and should be supported by a tree-based analysis or another analysis that shows these genes have greatest similarity to this particular human paralog.
Authors' response: Thank you for the suggestion. We have now clarified this by including an additional supplementary figure (now Supplementary Figure 10) of a phylogenetic tree that highlights the clustering of IFIT1 and IFIT3 for the species of interest.



The motifs mentioned in GBP1 and IFIT1: it should be clarified whether these motifs are "classical" linear motifs found in disordered regions (and possibly also annotated in databases such as the ELM database).

Authors' response: Thank you for the comment. In terms of IFTI1, the interaction motif is known to be in a structured helix at the c-terminal region of the protein. It is not thought to be a classical linear motif, and the ELM database does not pick up the IFIT interaction motif. For GBP1, the AlphaFold predictions for each species highlights that the AV1 motif is located in a beta turn, which is an ordered secondary structure. All three GBP1 sequences were also run through a disorder predictions program, DISOPRED, which further highlighted that this motif is in an ordered region.

If you'd like to take a look at the DISOPRED results:
Homo sapiens : http://bioinf.cs.ucl.ac.uk//psipred/&uuid=e1ae00ba-1580-11f0-849b-00163e100466
Pteropus alecto : http://bioinf.cs.ucl.ac.uk//psipred/&uuid=cdca99a8-1582-11f0-9bec-00163e100466
Eptesicus fuscus : http://bioinf.cs.ucl.ac.uk//psipred/&uuid=207b2bb8-1583-11f0-92a6-00163e100466

In Discussion:

In lines 632-633:
"These findings suggest an evolutionary adaption of the type I IFN response in bat species, which in turn may lead to a species-specific antiviral response in bats."
The mentioned findings do not necessarily suggest adaptation and/or species-specific antiviral response in bats. Rather, they suggest that there is rapid coding sequence evolution of Type I IFNs and their receptors, such that IFNs from various species cannot bind efficiently IFNARs and elicit a strong response. Universal IFN is an artificial construct that (rather weirdly) works on several species' IFNAR, but it is not directly related to species-adaptation in these genes.
Authors' response: Thank you for the comment. As part of trimming the manuscript to meet the word count restriction, the previous sentence has now been removed.

The following sentence (lines 675-676):
"This selection has been shown to be even higher in bats in comparison to other mammals" is phrased in an unclear manner – what do you mean by "even higher in bats"? Please clarify or rephrase.
Authors' response: Thank you for the comment. We have now clarified the sentence by editing it to the following: *Metagenomic analyses have demonstrated that immune genes, like ISGs are more likely to undergo selection, with bats experiencing a high rate of selection within immune genes compared to other mammals*[89]