EMBO reports

Interferon inhibits a model RNA virus via a limited set of inducible effector genes

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Dear Dr. Schoggins,

Thank you for the submission of your manuscript to EMBO reports. I have now received the reports from the three referees that were asked to evaluate your study, which can be found at the end of this message.

As you will see, the referees indicate that these findings are of interest. However, they have several comments, concerns, and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and all the referee concerns need to be addressed as indicated in the reports, I will not detail them here.

Given the constructive referee comments, I would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. Please contact me to discuss the revision (also by video chat) if you have questions or comments regarding the revision, or should you need additional time.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

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2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures (up to 8) and EV figures. Please upload these as separate, individual files upon re-submission.

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4) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an appropriate public database. If no primary datasets have been deposited, please also state this in a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

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Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability" section (placed after Materials & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study. This section is mandatory. As indicated above, if no primary datasets have been deposited, please state this in this section

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We now request the publication of original source data with the aim of making primary data more accessible and transparent to the reader. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (also for potential EV figures and all those in the final Appendix). Please also check that all the p-values are explained in the legend, and that these fit to those shown in the figure. Please provide statistical testing where applicable. Please avoid the phrase 'independent experiment', but clearly state if these were biological or technical replicates. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2, please show the data as separate datapoints without error bars and statistics. See also: http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

If n<5, please show single datapoints for diagrams.

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Title page - Abstract - Keywords - Introduction - Results - Discussion - Materials and Methods - Data availability section - Acknowledgements - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

In this manuscript, McDougal et al. use CRISPR-based loss-of-function screens to identify interferon stimulated genes (ISGs) that are able to restrict the alphavirus VEEV. They discover that only a small number of known ISGs are able to inhibit this virus, and that most of the IFN-induced suppression is dominated by proteins encoded by only three ISGs: IFIT1, IFIT3 and ZAP. They further show that this trio of ISGs had differing levels of anti-viral activity against other RNA viruses, suggesting there may be distinct combinations of ISGs required to restrict different viruses. They conclude that the type I interferon response to VEEV and potentially many other viruses, can be attributed to the effect of only a few particular ISGs acting in combination, rather than requiring concerted action of the many hundreds of ISGs that are induced in response to type I interferons.

Overall, the data presented in the manuscript are of high quality, the manuscript is well written and the study would be of great interest to the field. However, there a number of limitations which need to be addressed to allow these data to fully support the conclusions made.

Major comments:
1. The three ISGs described here have all been previously demonstrated to have antiviral activity against alphaviruses, and so this study is not reporting novel ISGs that inhibit VEEV. Instead, the key advance in knowledge would be the conclusion that these three are necessary and sufficient for restriction of VEEV. The study does not sufficiently address the relative contribution of each of the three in order to show that all three are required. In Figure 1D, silencing of IFIT1 has the greatest effect on loss of antiviral effect. As detailed in the Introduction of the manuscript, previous studies have shown that one gene can be responsible for the IFN-mediated viral restriction. The authors therefore have to show more convincingly that IFIT1 is not solely responsible for the restrictive effect by additionally including single gene knockouts for IFIT1, IFIT3 and ZCH3HAV1 in Figure 3A and/or 3B.

2. IFIT1 and IFIT3 can also be induced independently of IFNAR signalling via IRF3 and IRF7 (PMID: 30871003, PMID: 11991981, PMID: 23300459) and so induction of these genes can occur in a cell autonomous manner following viral infection. To support the conclusion that in this model the induction and therefore effects of IFIT1 and IFIT3 are mediated by IFN, the authors need to generate ZAP/IFIT3/IFIT1-targeted cells on an IFNAR-deficient background and repeat the infection in the presence and absence of IFN-β treatment of these cells, in addition to the other conditions included in Figure 3B.

3. An MOI of 5 or 10 VEEV is used in this study, indicating each cell is infected with multiple virions. The choice of MOI is not explained in the text. The authors should include a dose titration of virus in WT and ZAP/IFIT3/IFIT1-targeted cells to determine if the same effect is seen when lower MOIs are used and should also justify in the text the choice of MOI used for other experiments.

4. Figure 2 shows that IFIT1, IFIT3 and ZCH3HAV1 expression is higher at 6 h than 24 h post IFN-β treatment but there are more ISGs in total at 24 h, suggesting that these three are "early ISGs". All three have a role in blocking viral translation, therefore considering the kinetics of their role in VEEV restriction would enhance the manuscript. Only 1 time point was used for each virus (4.5 - 5 h.p.i. for VEEV-GFP and 12 h.p.i. for VEEV). Additional experiments infecting ZAP/IFIT3/IFIT1-targeted cells at additional timepoints are required.

Minor points:
- Line 122: an explanation of why these 10 genes in particular were chosen for validation is needed
- Line 146: padj should be < 0.05
- Data Accessibility section with a link to the RNAseq data is missing from the manuscript

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Referee #2:

McDougal et al show that only a small number of interferon-stimulated genes (three, in fact) contribute to the majority of the IFN-mediated inhibition of the alphavirus, VEEV. The authors show that these three ISGs are only a minor component at the mRNA level of the overall IFN response, though contribute to the majority of the inhibition. The results argue that the interferon response is dominated by just a few genes, and that this set of genes is likely different for different viruses (since the same set of three were not major players even against another alphavirus). This is a very fine manuscript with a clear definitive result that will

be of much interest to the field.

I have only very minor comments.

1. Line 67: I would also add that over-expression assays with different ISGs often over-emphasize effect sizes or even cause antiviral effects that do not exist at physiological levels of the ISG. I think some of the confusion in the literature of how many ISGs are antiviral for a given virus is that many different ISGs are antiviral in over-expression assays, but that may not reflect the actual effects of the endogenous expression levels.
2. Line 120: Please mention here that the screens were done in U2OS cells, rather than only in the Methods. The authors should also justify why they chose this cell line for the screen. As they are aware, the choice of cell line is going to influence what ISGs are expressed which will affect the CRISPR screen. (although it is reassuring to see that the same set of genes also accounts for the IFN response in another cell line they tested).
3. LINE 205 and Discussion: I was surprised to see such a difference in response to these three genes between the different alphaviruses tested. I think a little more speculation in the Discussion is warranted. Could this be due to the differences in mechanism of host shut off between alphaviruses? Are each of these viruses equally sensitive to IFN to start with?

Referee #3:

Major points:

Although this research goes a long way to answering important questions in the ISG field, and the experiments undertaken have required a great deal of technical skill; its physiological relevance is very low; and perhaps this work might be better suited for a more specialty journal. This is mainly due to the fact that the authors have excluded any examination of type III interferon; a major antiviral cytokine in restriction of viruses in epithelial cells; which are used throughout this manuscript.

Although briefly discussed as a caveat in the discussion; type III interferon is a major antiviral interferon known to restrict Alphaviruses; and indeed is more dominant at restricting the alphavirus, CHIKV than type I IFN in U-2 OS cells, the main cell type in this manuscript (Pott et al, Emerg Microbes Infect; 2021). I understand that the role of ISGs in restriction of viruses is a complex process, and although it is interesting that a very small subset of ISGs is involved in restricting VEEV infection by type I IFN (and potentially some other alphaviruses), having a throw away line at the end of the discussion concerning a major player in the antiviral response of epithelial cells (Which are used in this study) is not sufficient acknowledgement of what may be occurring at the physiological level.

Minor suggestions:

Line 152; readers outside of the ISG field may find it confusing when you jump from the gene name (ZC3HAV1) to ZAP; I would include something here to explain this.

Line 209; I find this statement very confusing:

'Together, our data suggest that IFN-mediated restriction of RNA viruses may 210 be: 1.) completely independent of ZAP, IFIT3, and IFIT1, 2.) largely independent of ZAP, IFIT3, 211 IFIT1, which play minor roles in viral restriction, 3.) largely dependent on ZAP, IFIT3, and IFIT1.'

How can 1. Be correct? You have shown that at least some RNA viruses do require these three ISGs for IFN mediated restriction?

McDougal et al. Response to Reviewer comments

Referee #1:

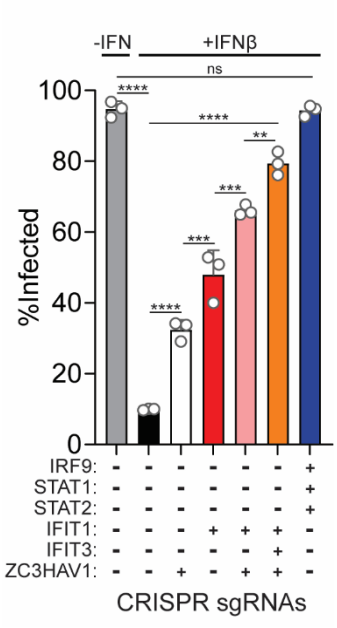
In this manuscript, McDougal et al. use CRISPR-based loss-of-function screens to identify interferon stimulated genes (ISGs) that are able to restrict the alphavirus VEEV. They discover that only a small number of known ISGs are able to inhibit this virus, and that most of the IFN-induced suppression is dominated by proteins encoded by only three ISGs: IFIT1, IFIT3 and ZAP. They further show that this trio of ISGs had differing levels of anti-viral activity against other RNA viruses, suggesting there may be distinct combinations of ISGs required to restrict different viruses. They conclude that the type I interferon response to VEEV and potentially many other viruses, can be attributed to the effect of only a few particular ISGs acting in combination, rather than requiring concerted action of the many hundreds of ISGs that are induced in response to type I interferons.

Overall, the data presented in the manuscript are of high quality, the manuscript is well written and the study would be of great interest to the field. However, there a number of limitations which need to be addressed to allow these data to fully support the conclusions made.

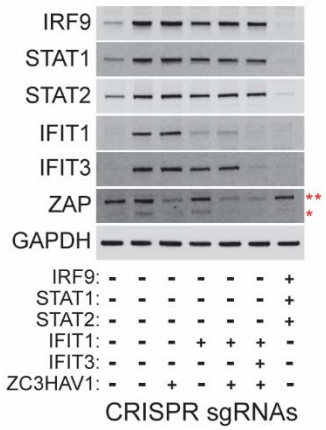
Major comments:
1. The three ISGs described here have all been previously demonstrated to have antiviral activity against alphaviruses, and so this study is not reporting novel ISGs that inhibit VEEV. Instead, the key advance in knowledge would be the conclusion that these three are necessary and sufficient for restriction of VEEV. The study does not sufficiently address the relative contribution of each of the three in order to show that all three are required. In Figure 1D, silencing of IFIT1 has the greatest effect on loss of antiviral effect. As detailed in the Introduction of the manuscript, previous studies have shown that one gene can be responsible for the IFN-mediated viral restriction. The authors therefore have to show more convincingly that IFIT1 is not solely responsible for the restrictive effect by additionally including single gene knockouts for IFIT1, IFIT3 and ZCH3HAV1 in Figure 3A and/or 3B.

We thank the reviewer for the suggestion. To address this comment, we carried out new experiments in which we CRISPR targeted IFIT1 alone, ZC3HAV1 alone, IFIT1 + ZC3HAV1 together, or ZC3HAV1 + IFIT1 + IFIT3 together. We did not include IFIT1 + IFIT3 or ZC3HAV + IFIT3, as IFIT3 has been shown to heterodimerize with IFIT1 to enhance antiviral activity (PMID: 2952552). CRISPR-targeted cells were infected cells with VEEV-GFP and infectivity was quantified by flow cytometry. A stronger antiviral effect was observed with two-gene CRISPR-based targeting (ZC3HAV1 and IFIT1) than either gene alone, and the strongest effect was observed with all 3 genes were targeted in combination (see below **Response Figure 1A**). We confirmed successful CRISPR-targeting by western blotting, as a reduction in protein product abundance of targeted genes was observed for all conditions (see below **Response Figure 1B)**. These data indicate that IFIT1 is not solely responsible for the restrictive activity. We have included these data as **Figure 3C** and **Expanded View Figure 3C** in the revised manuscript.

A



B



Response Figure 1.

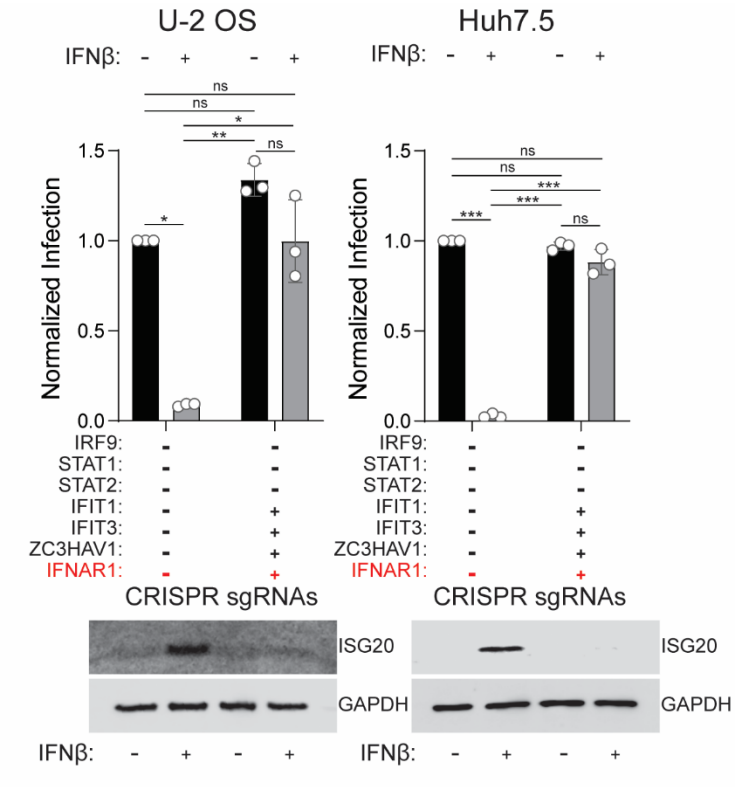
A. Effects of CRISPR-based single, double, or triple gene inactivation on VEEV infection (MOI of 5 for 5 hrs) in U-2 OS cells with or without IFNβ pretreatment (20U/mL for 24 hrs). n=3 biological replicates, One-way ANOVA with Dunnett's test.

B. Western blot showing reduced protein abundance in CRISPR-targeted cells from A (above). Red asterisks denote long (**) and short (*) isoforms of ZAP.

2. IFIT1 and IFIT3 can also be induced independently of IFNAR signalling via IRF3 and IRF7 (PMID: 30871003, PMID: 11991981, PMID: 23300459) and so induction of these genes can occur in a cell autonomous manner following viral infection. To support the conclusion that in this model the induction and therefore effects of IFIT1 and IFIT3 are mediated by IFN, the authors need to generate ZAP/IFIT3/IFIT1-targeted cells on an IFNAR-deficient background and repeat the infection in the presence and absence of IFN-β treatment of these cells, in addition to the other conditions included in Figure 3B.

We appreciate the insightful suggestion. We performed the requested experiment by inactivating IFNAR1 via CRISPR in the background of the ZAP/IFIT3/IFIT1-targeted cells in both U-2 OS and Huh7.5 cells. Cells were treated with IFN or mock-treated and then infected with VEEV-GFP at an MOI of 1 for approximately 1 viral replication cycle. An MOI of 1 was chosen

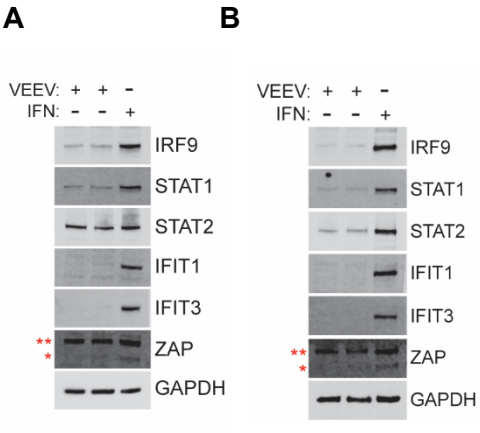
so there would be sufficient uninfected cells that might become infected were any IFN-independent restriction factors ablated that prevented VEEV infection. While we observed a slight increase in the ratio of infected cells in ZAP/IFIT3/IFIT1/IFNAR1-targeted cells without IFN treatment compared to non-targeting controls, this was not statistically significant (see below **Response Figure 2, top)**. We confirmed that these ZAP/IFIT3/IFIT1/IFNAR1-targeted cells were unable to response to IFN by western blot, as lysates from these cells treated with IFN did not result in detectable levels of ISG20 while control cells treated with IFN resulted in ISG20 induction (see below **Response Figure 2, bottom)**. We conclude that ZAP, IFIT3, and IFIT1 play a significant role in the IFN-mediated restriction of VEEV (Figure 3A-3C) and a very minor role in the IFN-independent restriction of VEEV. These new data have been included as **Expanded View Figure3D** of the revised manuscript.



Response Figure 2.

Infection of CRISPR-targeted U-2 OS (Left, top) or Huh7.5 cells (Right, top) that were infected with an MOI of 1 VEEV-GFP with and without pretreatment of IFNβ (24 h treatment, 20U/mL for U-2 OS and 100U/mL for Huh7.5 cells). Western blot of ISG20 from lysates of "quadruple-targeted" U-2 OS (Left, bottom) or Huh7.5 cells (Right, bottom) with and without pretreatment of IFNβ (24 h treatment, 20U/mL for U-2 OS and 100U/mL for Huh7.5 cells).

To additionally address the possibility that ISGs are being upregulated directly by viral infection in the absence of IFN, we infected U-2 OS cells with identical conditions to those used in experiments included in the manuscript and performed western blotting. Infection of U-2 OS cells with VEEV-GFP (see below **Response Figure 4A**).) or the VEEV TC-83 untagged virus (see below **Response Figure 4B**).) at the timepoints previously tested did not result in an observable increase of the protein abundance of IFIT1, IFIT3, or ZAP. We have included these data as **Expanded View Figure 3E** (VEEV-GFP) and **Expanded View Figure 5A** (untagged VEEV).



Response Figure 4.

A. Western Blot from lysates of U-2 OS cells after 5 h infection with VEEV-GFP or IFNβ treatment (20U/mL for 24 h). Red asterisks denote long (**) and short (*) isoform of ZAP.

B. Western Blot from lysates of U-2 OS cells after 12 h infection with VEEV TC-83 or IFNβ treatment (20U/mL for 24 h). Red asterisks denote long (**) and short (*) isoform of ZAP.

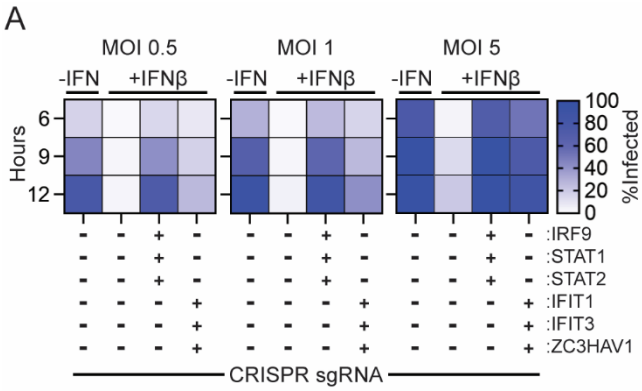
3. An MOI of 5 or 10 VEEV is used in this study, indicating each cell is infected with multiple virions. The choice of MOI is not explained in the text. The authors should include a dose titration of virus in WT and ZAP/IFIT3/IFIT1-targeted cells to determine if the same effect is seen when lower MOIs are used and should also justify in the text the choice of MOI used for other experiments.

4. Figure 2 shows that IFIT1, IFIT3 and ZCH3HAV1 expression is higher at 6 h than 24 h post IFN-β treatment but there are more ISGs in total at 24 h, suggesting that these three are "early ISGs". All three have a role in blocking viral translation, therefore considering the kinetics of their role in VEEV restriction would enhance the manuscript. Only 1 time point was used for each virus (4.5 - 5 h.p.i. for VEEV-GFP and 12 h.p.i. for VEEV). Additional experiments infecting ZAP/IFIT3/IFIT1-targeted cells at additional timepoints are required.

We thank the reviewer for these two comments, which we have addressed together. For comment #3, we have added the following sentence on lines 118-119 for justifying the choice of higher MOIs in our experiments, “[An MOI of 5 or 10] was chosen to allow for a high signal to noise ratio in which nearly all cells become infected without IFN pretreatment, yet almost no cells are infected with IFN-pretreatment.”

To address viral dose (Comment #3) and time point (Comments #4), we infected cells with 5-20 times less virus than previous VEEV-GFP experiments (MOIs of 0.5 and 1). We infected control or ZAP/IFIT3/IFIT1-targeted cells for 6h, 9h, and 12h (see below **Response Figure 5A**). At the earliest timepoint (6 h), *the majority of the IFN-mediated restriction of VEEV-GFP was mediated by ZAP, IFIT3, and IFIT1 at all MOIs*. At later timepoints, ZAP, IFIT3, and IFIT1 still played a major and significant role in the restriction of VEEV-GFP. However, at lower MOIs there was still a notable IFN-mediated inhibition in the triple knockout cells at later timepoints (9 and 12h), which represents more than one replication cycle. This could be due to several factors: 1) We have incomplete CRISPR-based targeting of ISGs in our cells (Fig EV3A-C and Fig 5B, bottom). Thus, any inhibition from residual “dominant” antiviral effectors may be amplified by multiple viral replication cycles, 2) Other ISGs may play minor roles in viral restriction compared to the “dominant” antiviral ISGs we have identified here. 3) One or more antiviral effectors acting during assembly or egress may have roles in this assay, but were missed in the single-cycle assay of the original screen. This minor restriction may also be amplified by multiple viral replication cycles. Furthermore, in Figure 5C (formerly Figure 5B) we tested the ability of other previously described anti-alphavirus effectors contribute to this residual IFN-mediated restriction

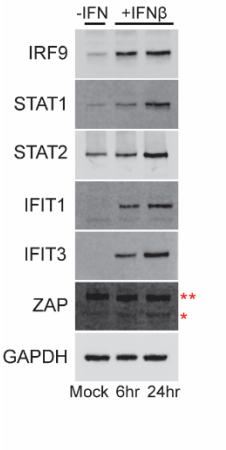
of VEEV (which could fall under scenario 2 or 3 listed above). We found that none previously described anti-alphavirus ISGs tetherin, viperin, IFITM3, or ISG20 had an effect on the production of VEEV. This further suggests that the residual IFN-mediated restriction of VEEV in ZAP/IFIT3/IFIT1-targeted cells might be due to incomplete CRISPR-targeting as noted above. We have added this new data as **Figure 5A** of the revised manuscript, as well as additional Discussion on the speculations mentioned above.



Response Figure 5.

A. Heatmaps showing the ratio of infected cells at 6, 9, and 12 h of infection with VEEV-GFP. U-2 OS control, IRF9/STAT1/STAT2-targeted, or ZAP/IFIT3/IFIT1-targeted cells were treated with or without IFNβ (20U/mL for 24 hrs) and infected with an MOI of 0.5 (left), 1 (middle), or 5 (right) of VEEV-GFP. Infection was quantified by flow cytometry. n=3 biological replicates.

As the reviewer pointed out, the mRNA expression at 6h post-IFN treatment is higher than at 24h post IFN-treatment (Figure 2D). We were therefore interested in determining the kinetics of these ISG-effector products, which perform the antiviral action, after a 6 or 24h IFNβ treatment. We observed that the abundance of ZAP, IFIT3, and IFIT1 protein were all higher at 24h post-IFN treatment compared to 6h by western blotting (See below **Response Figure 6**). Our data suggest that while the mRNA levels of ZAP, IFIT1, and IFIT3 are waning by 24h, there is a greater accumulation of protein at 24h. This 24h IFN pretreatment is the timepoint used in all infection experiments. We have added this new data as **Figure 2E** of the revised manuscript.



Response Figure 6.

A. Western Blots from lysates of U-2 OS cells treated with IFNβ for 6 and 24 hrs. Antibodies were used to probe for IFN-induced CRISPR screening hits.

Minor points:

- Line 122: an explanation of why these 10 genes in particular were chosen for validation is needed

We have added the following sentence on lines 129-130, “These 10 genes were chosen based on known or predicted functions in immunity, post-translational modification, or reported differential expression after IFN-treatment.”

- Line 146: padj should be < 0.05

We thank the reviewer for pointing this mistake out. This has been changed (line 155).

- Data Accessibility section with a link to the RNAseq data is missing from the manuscript

The RNA-Seq and CRISPR Screening data have been all deposited to the GEO database with the SuperSeries accession number GSE233197.
RNA-Seq data: Gene Expression Omnibus, accession GSE233003. CRISPR screening data: Gene Expression Omnibus, accession GSE233195 (genome-wide screen) and accession GSE233196 (ISG-targeted screen). All will be publicly available on May 26, 2023.

Referee #2:

McDougal et al show that only a small number of interferon-stimulated genes (three, in fact) contribute to the majority of the IFN-mediated inhibition of the alphavirus, VEEV. The authors show that these three ISGs are only a minor component at the mRNA level of the overall IFN response, though contribute to the majority of the inhibition. The results argue that the interferon response is dominated by just a few genes, and that this set of genes is likely different for different viruses (since the same set of three were not major players even against another alphavirus). This is a very fine manuscript with a clear definitive result that will be of much interest to the field.

I have only very minor comments.

1. Line 67: I would also add that over-expression assays with different ISGs often over-emphasize effect sizes or even cause antiviral effects that do not exist at physiological levels of the ISG. I think some of the confusion in the literature of how many ISGs are antiviral for a given virus is that many different ISGs are antiviral in over-expression assays, but that may not reflect the actual effects of the endogenous expression levels.

We appreciate the reviewer pointing out this fact. The following sentence has been added to the manuscript on lines 67-68, “In addition, overexpression may lead to non-physiological levels of expression that can result in artificial inhibition of viral infection.”

2. Line 120: Please mention here that the screens were done in U2OS cells, rather than only in the Methods. The authors should also justify why they chose this cell line for the screen. As they are aware, the choice of cell line is going to influence what ISGs are expressed which will affect the CRISPR screen. (although it is reassuring to see that the same set of genes also accounts for the IFN response in another cell line they tested).

We thank the reviewer for their suggestions. We have added the following sentence to justify the use of U-2 OS cells and clearly communicate the use of this cell type for the screen on lines 115-116, “U-2 OS cells were chosen for the screen due to their high permissibility to VEEV as well as robust inhibition of VEEV after IFN pretreatment.”

3. LINE 205 and Discussion: I was surprised to see such a difference in response to these three genes between the different alphaviruses tested. I think a little more speculation in the Discussion is warranted. Could this be due to the differences in mechanism of host shut off between alphaviruses? Are each of these viruses equally sensitive to IFN to start with?

We share the surprise and interest with the reviewer at this observation. VEEV and ONNV are similarly sensitive to IFN pretreatment (Figure 3A vs Figure 4A). We speculate that this difference in restriction by ZAP, IFIT3, and IFIT1 is due to differences in the features of the viral RNA between ONNV and VEEV. We have added the following sentence to the discussion as requested by the reviewer, “As ZAP, IFIT1, and IFIT3 recognize distinct features of viral RNA, we speculate that differences in ZAP, IFIT3, and IFIT1-mediated restriction of ONNV and VEEV may be due to differences in abundance or distribution of CpG dinucleotides (which could affect ZAP-mediated restriction) and/or differences in 5’ RNA secondary structure or capping of viral RNA (which could affect restriction by IFITs).” In addition, we hypothesize that VEEV and ONNV would have similar mechanisms and abilities in terms of host shut off. A recent study shows the ability of VEEV and CHIKV (as well as other alphaviruses) to perform host translational shut-off via eEF2 phosphorylation through viral protein nsp2 (PMID: 36848386). While we were unable to find any studies that specifically evaluated host shut-off by ONNV, we hypothesize that ONNV would induce host shut-off similarly to other alphaviruses.

Referee #3:

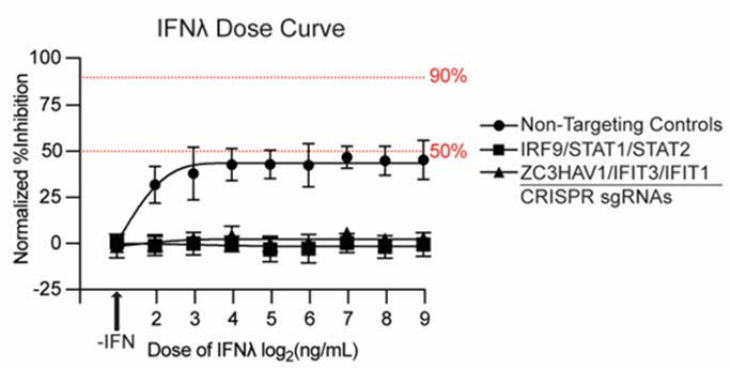
Major points:

Although this research goes a long way to answering important questions in the ISG field, and the experiments undertaken have required a great deal of technical skill; its physiological relevance is very low; and perhaps this work might be better suited for a more specialty journal. This is mainly due to the fact that the authors have excluded any examination of type III interferon; a major antiviral cytokine in restriction of viruses in epithelial cells; which are used throughout this manuscript.

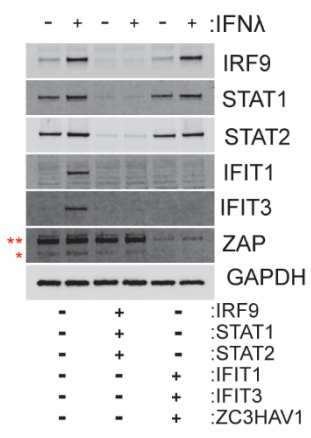
Although briefly discussed as a caveat in the discussion; type III interferon is a major antiviral interferon known to restrict Alphaviruses; and indeed is more dominant at restricting the alphavirus, CHIKV than type I IFN in U-2 OS cells, the main cell type in this manuscript (Pott et al, Emerg Microbes Infect; 2021). I understand that the role of ISGs in restriction of viruses is a complex process, and although it is interesting that a very small subset of ISGs is involved in restricting VEEV infection by type I IFN (and potentially some other alphaviruses), having a throw away line at the end of the discussion concerning a major player in the antiviral response of epithelial cells (Which are used in this study) is not sufficient acknowledgement of what may be occurring at the physiological level.

We appreciate the reviewer pointing out the importance of Type III IFNs, which we indeed did not adequately address. To examine the role of Type III IFNs in the context of VEEV infection as well as the role of ZAP, IFIT3, and IFIT1 in Type III IFN-mediated viral restriction, we performed an IFNλ dose response in Non-targeting control, IRF9/STAT1/STAT2, and ZAP/IFIT3/IFIT1-targeted U-2 OS cells followed by an infection with VEEV-GFP (MOI of 5 for 5 h). Interestingly, over a range of IFN concentrations, IFNλ was not as effective at restricting VEEV in U-2 OS cells as IFNβ. At the highest dose of 500ng/ml, IFNλ treatment was only able to restrict infection by approximately 45%, as compared to near complete suppression by IFNβ (see below **Response Figure 7A**). Almost all of the restriction observed by IFNλ was mediated by ZAP, IFIT3, and IFIT1 in these experiments, as no IFNλ-mediated restriction of VEEV-GFP was observed in cells in which ZC3HAV1, IFIT3, and IFIT1 were silenced by CRISPR. We also observed appreciable induction of ZAP, IFIT3, and IFIT1 after IFNλ treatment (see below **Response Figure 7B**). Thus, while there may be subtle differences between type I versus type III IFN sensitivity between CHIKV (Pott et al paper) and VEEV (our study) in U-2 OS cells, our new data indeed support the reviewer's position that type III inhibits VEEV in cells of epithelial origin and that the inhibition is mediated by IFIT1, IFIT3, and ZAP. These new data improve the physiological relevance of our study and have been added to the revised manuscript as **Figure 3D, right** and **Expanded View Figure 3F**.

A



B



Response Figure 7.

A. Dose-curve of IFNλ pretreatment 2-fold dilutions from 500 ng/mL to 3.9 ng/mL for 24 hrs) demonstrating inhibition of VEEV infection (MOI of 5 for 5 hrs) in control (circles), IRF9/STAT1/STAT2-targeted (squares), or ZAP/IFIT3/IFIT1-targeted (triangles) U-2 OS cells.

B. Western Blot from lysates of U-2 OS CRISPR-targeted or control cells after 24 h treatment 500ng/mL of IFNλ. Red asterisks denote long (**) and short (*) isoform of ZAP.

Minor suggestions:

Line 152; readers outside of the ISG field may find it confusing when you jump from the gene name (ZC3HAV1) to ZAP; I would include something here to explain this.

We acknowledge that the switch between "*ZC3HAV1"* and "ZAP" may be confusing. We consistently use *ZC3HAV1* when referring to the gene (CRISPR-targeting, etc.) and ZAP when referring to the protein-product of the gene that exhibits antiviral activity. We have attempted to clarify this in the manuscript by adding, "ZAP (encoded by the gene *ZC3HAV1*)" on line 163 when "ZAP" is first introduced.

Line 209; I find this statement very confusing:

'Together, our data suggest that IFN-mediated restriction of RNA viruses may 210 be: 1.) completely independent of ZAP, IFIT3, and IFIT1, 2.) largely independent of ZAP, IFIT3, 211 IFIT1, which play minor roles in viral restriction, 3.) largely dependent on ZAP, IFIT3, and IFIT1.'

How can 1. Be correct? You have shown that at least some RNA viruses do require these three ISGs for IFN mediated restriction?

We apologize for the confusing language used in the original manuscript and thank the reviewer for pointing this out. We have rewritten the sentence to clarify this comment as shown below:

"Together, our data suggest that IFN-mediated restriction of any given RNA virus may be: 1.) completely independent of ZAP, IFIT3, and IFIT1, exemplified by ZIKV (Figure 4A, right) 2.) partially dependent on ZAP, IFIT3, IFIT1, which play minor roles in viral restriction, exemplified by VSV (Figure 4B, right) 3.) largely dependent on ZAP, IFIT3, and IFIT1, exemplified by VEEV (Figure 3A)."

Dear Dr. Schoggins,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the three referees that I asked to re-evaluate your study, you will find below. As you will see, the referees now fully support the publication of your study in EMBO reports. Referee #1 has a final point I ask you to check.

Moreover, I have these editorial requests I ask you to address in a final revised manuscript.

- Please remove the section 'Topics' from the title page.

- In the "Data Availability section" (DAS) please add direct links to the datasets and make sure these are public latest upon publication of the study.

- Please name the EV Figures EV1 and EV2 and change the callouts and the legends accordingly. Moreover, please make sure that all figure panels are called out, that they are called out separately and sequentially. Presently, there seems to be no callout for panel EV3F (should be EV1F). Moreover, the callout for EV3D (EV1D) should come after the one for EV3C (EV1C). Finally, the label A in Figure EV5 (EV2) should be removed, as there is only one panel. Please check.

- Tables S1 and S2 are datasets. Please rename these to Dataset EV1 and Dataset EV2 and upload them as full excel files (as dataset files), with names and legends on the first TAB. Please change their callouts in the manuscript text. Like this, we do not need the Appendix file.

- Finally, please find attached a word file of the manuscript text (provided by our publisher). Please prepare your final manuscript file using the attached file as basis and with track changes, in order that we can see any modifications done.

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- two to four short (!) bullet points highlighting the key findings of your study (not more than two lines each).
- a schematic summary figure (in jpeg, png or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

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Best,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

The authors have sufficiently addressed the comments from myself and the other reviewers. The additional experiments strengthen the manuscript and the data more fully support the conclusions made so I recommend this manuscript for publications. The authors will need to correct the error in the labelling of Response Figure 4 (now Figure EV3E and EV5A), the first lane of both should be -VEEV.

Referee #2:

The authors have done a very good job responding to the reviewer comments.

Referee #3:

The revised manuscript is improved, and has sufficiently answered my major criticism from the original version.

The authors have addressed all minor editorial requests.

Dr. John Schoggins
UT Southwestern Medical Center
Microbiology
6000 Harry Hines Blvd
Dallas, TX 75390
United States

Dear Dr. Schoggins,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
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  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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