

HTLV-1 subverts the innate immune effector gene IRF7 by viral HBZ protein for oncogenesis

Corresponding Author: Dr Guangyong Ma

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 study examined the role of IRF7 in HTLV-1 infection and related diseases.

They showed that expression of HTLV-1 in zebrafish ECMs caused pronounced developmental defects and that the HBZ protein and not Tax1 was responsible for the defects. RNA-SEQ on HBZ expressing ESCs showed that HBZ activated innate immune while it dysregulated metabolic pathways suggested to account for the developmental changes in the zebrafish. The authors show that HBZ enhances the expression of IRF7 at a transcriptional and post transcriptional level in a variety of cell lines and enhanced activation of ISRE by IRF7. HBZ promoted IRF7 phosphorylation at S477 via TBK1 and not IKKE and promoted the nuclear localisation of phosphorylated IRF7. Analysis of the scRNA-seq data showed the IFN-I pathway was inactive in HBZ expressing zebrafish ESCs and ATLL clinical samples. The authors further showed that HBZ interacts with IRF7 via its bZip domain and this interaction interfered with the interaction between IRF7 and IRF3, suggesting that this inhibits IFN pathway. IRF7 was shown to promote the proliferation and survival of HTLV-1 infected cells and promoted the tumorigenicity of ED and TLOm-1 cells suggesting it acts as an oncogene in ATLL. The authors showed that STAT5B is involved in IRF7 oncogenesis.

The most noteworthy results presented in the manuscript relates to the demonstration that IRF7 may have an oncogenic role in ATLL. This original research is significant and will be of interest to the HTLV-1 research community. The work supports the conclusions drawn and methods are sufficiently described.

Comments

Figure 3

-The authors should show the effect of HBZ on IRF7 activation of ISRE in cells other than HeLa cells e.g. T cells (Figure 3A). Since IRF3 plays a critical role in the induction of the IFN pathway, analysis of the effect of HBZ on IRF3 ISRE activation would be warranted. Narulla et al. showed that HBZ inhibits IRF3 activation of ISRE containing reporters. Could the authors comment on the possibility that the inhibitory effect of HBZ on IRF3 counterbalances the enhanced effect it has on IRF7 activation resulting in no overall effect on the IFN pathway?

-HBZ displays a distinctive punctate localization in the nuclei of cells. This localization is not apparent in Figure 3B. It is also not clear whether IRF7 and HBZ colocalized in the nuclei of these cells. Can the authors address this

-"C" needs to be added to this figure

-Did the authors analyse phosphorylation of sites other than Ser477 in IRF7.

Figure 4

-Authors show that the binding of HBZ to IRF7 inhibits the binding of IRF7 to IRF3 suggesting that this may prevent the induction of IFN-I. Can the authors comment on a mechanism. Does HBZ bind the same domain as IRF3 on IRF7?

Reviewer #2

(Remarks to the Author)

This study revealed a new regulatory mechanism of HBZ on promoting IRF7-mediated HTLV-1 virus growth and antagonizing type-I interferon antiviral signaling. However, the data quality of this manuscript can be further improved, and comments/suggestions are listed below:

In Figure 2, the Authors clearly demonstrated that HBZ was enriched upstream of the TSS of IRF7. Authors should consider the use of the promoter (compare the wild-type with an IRF7 putative binding site mutant) reporter assay in HeLa, to further demonstrate that the “specificity” of such binding/enrichment is a major contributor to the IRF7 protein expression.

In Figure 3C, how does HBZ exactly promote IRF7 Ser477 phosphorylation? Is it due to the induction of TBK1 expression by HBZ as described in the manuscript, leading to more phosphorylation of IRF7? Was the induction of “excess” TBK1 necessary for the IRF7 activation (phosphorylation)? In a normal situation, the abundance of kinases is not the limiting factor. Protein expression of TBK1 can be included together with Figure 3G.

In Figure 4D, the authors claim that HBZ binding to IRF7 inhibits IRF3-IRF7-mediated interferon signaling. First, authors can indicate whether ionic binding or hydrogen binding is predicted for HBZ-IRF7, including the distance between the predicted interaction and the overall binding energy, so that the authors have an idea of how strong the binding is predicted by AlphaFold.

In Figure 4E-G, the IP data showed the association of overexpressed IRF7 and HBZ, but not the direct interaction between them. Since this is the key hypothetical model of HBZ-mediated interferon antagonism, a direct interaction between IRF7 and HBZ is required. The use of purified recombinant proteins (including the bZIP mutant) for direct protein-protein interactions and binding affinity (Biacore, ITC, etc.) should be measured. The DNA contamination in the recombination protein should be excluded since the bZIP domain of HBZ is responsible for the binding.

In Figure 4, the authors claim that HBZ interferes with the IRF3-IRF7 interaction to prevent IFN-I activation using a non-induced zebrafish model. Further improvement of data quality is needed.

In Figure 4A, if no induction of the IFN pathway, or basal expression of IFN-I/ISG is low/absent in normal zebrafish ESC, how can “IFN pathway was inactive in HBZ-expressing zebrafish ESC, line 179” be concluded?

Referring to Figure 4B, “Analysis of the scRNA-seq data showed that the IFN-I pathway was not active either in ATLL clinical samples”. Only limited information was provided (line 180), and no guidance was given to help the reader interpret the scRNA-seq data.

In Figure 4H, IFN should be activated so that the HBZ effect can be observed.

In Figure 5, how the “ATLL cells stably expressing IRF7-targeting shRNA” be generated, if the IRF7 knockdown greatly impaired cell proliferation (Fig. 5B) and increased apoptosis (Fig. 5C) in ATLL cell lines. It is recommended to include a control: an expression plasmid for IRF7 with the shRNA targeting sequence mutated. This control should be added in the experiments described in Figure 5.

Reviewer #3

(Remarks to the Author)

In this study, Yuan et al. reported on the activation of IRF7 by HTLV-1 oncoprotein HBZ. The findings are of great interest as IRF7 was initially discovered as an oncoprotein activated by EBV oncoprotein LMP1. Multiple lines of evidence have been presented to make the point. However, the paper could be revised to focus more on the key finding. Data such as Figure 1 might be moved to supplementary. Several mechanisms for HBZ perturbation of IRF7 have been suggested. This is not surprising since viral oncoproteins are commonly very pleiotropic, but it will still be helpful if one key mechanism is characterized more thoroughly. Generally, the novel and interesting findings substantially advance the field.

- 1) Figures 1B and 1C: It appears that the same data were used twice. The two figures should be combined.
- 2) Figures 2A and 2B: Please specify the scRNA-seq cluster names.
- 3) Figures 2D and 4H: Does IRF7 drive NF- κ B activation?
- 4) Figure 2J: Can CRISPR knockout influence mRNA levels?
- 5) Figure 3: It will be of interest to verify whether HBZ alters IRF3 nuclear translocation.
- 6) Figure 3D: The observed increase in phosphorylated IRF7 (pIRF7) could simply reflect higher IRF7 levels.
- 7) Figure 3I: TBK1 detection should be confirmed by Western blotting. The result is weak.
- 8) Figure 4G: Immunoprecipitation of endogenous proteins might provide more physiologically relevant insights.
- 9) Does HBZ activate STAT5B? Additionally, is STAT5B activation observed in HTLV-1-infected cells?
- 10) Lines 189-90: “It seems the activation of IRF7 in HTLV-1 infected cells did not necessarily lead to the activation of IFN-I, probably owing to the presence of HBZ.” More experiments should be carried out to test it out. Integrity of IFN-I production

pathway in the cell line should be verified by transfecting RNA mimic poly (I:C) or DNA mimic VACV70 mer to activate RNA or DNA sensing pathway. Moreover, they should test if HBZ suppresses IFN production pathway by comparing IFN-I levels in HBZ-overexpressing cell with activated IFN production pathway.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

All the comments were fully addressed and supplemented with additional data from the suggested experiments. The revised manuscript is in good shape.

Reviewer #3

(Remarks to the Author)

All concerns raised in the previous round of review have been addressed satisfactorily. The paper is much improved and ready for publication.

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This study examined the role of IRF7 in HTLV-1 infection and related diseases. They showed that expression of HTLV-1 in zebrafish ECMs caused pronounced developmental defects and that the HBZ protein and not Tax1 was responsible for the defects. RNA-SEQ on HBZ expressing ESCs showed that HBZ activated innate immune while it dysregulated metabolic pathways suggested to account for the developmental changes in the zebrafish. The authors show that HBZ enhances the expression of IRF7 at a transcriptional and post transcriptional level in a variety of cell lines and enhanced activation of ISRE by IRF7. HBZ promoted IRF7 phosphorylation at S477 via TBK1 and not IKKE and promoted the nuclear localisation of phosphorylated IRF7. Analysis of the scRNA-seq data showed the IFN-I pathway was inactive in HBZ expressing zebrafish ESCs and ATLL clinical samples. The authors further showed that HBZ interacts with IRF7 via its bZip domain and this interaction interfered with the interaction between IRF7 and IRF3, suggesting that this inhibits IFN pathway. IRF7 was shown to promote the proliferation and survival of HTLV-1 infected cells and promoted the tumorigenicity of ED and TLOm-1 cells suggesting it acts as an oncogene in ATLL. The authors showed that STAT5B is involved in IRF7 oncogenesis.

The most noteworthy results presented in the manuscript relates to the demonstration that IRF7 may have an oncogenic role in ATLL. This original research is significant and will be of interest to the HTLV-1 research community. The work supports the conclusions drawn and methods are sufficiently described.

Author Response:

We greatly appreciate your insightful comments on our manuscript.

Comments

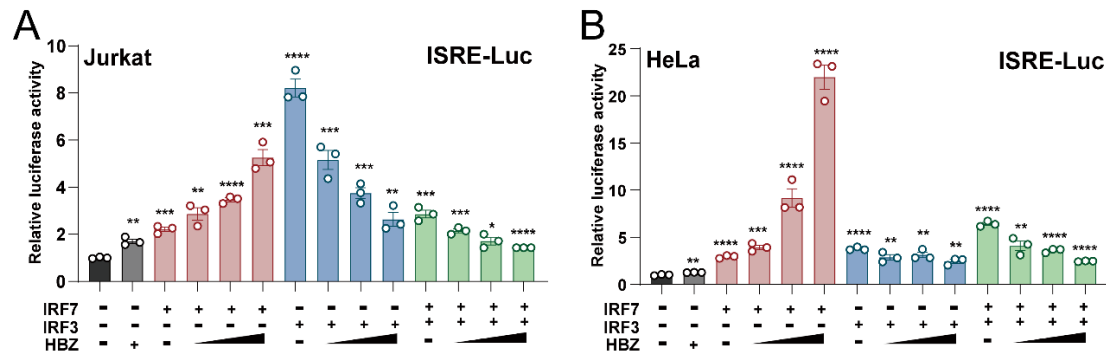
Figure 3

-The authors should show the effect of HBZ on IRF7 activation of ISRE in cells other than HeLa cells e.g. T cells (Figure 3A). Since IRF3 plays a critical role in the induction of the IFN pathway, analysis of the effect of HBZ on IRF3 ISRE activation would be warranted. Narulla et al. showed that HBZ inhibits IRF3 activation of ISRE containing reporters. Could the authors comment on the possibility that the inhibitory effect of HBZ on IRF3 counterbalances the enhanced effect it has on IRF7 activation resulting in no overall effect on the IFN pathway?

Author Response:

As suggested, Jurkat T cells were used to confirm the effect of HBZ on IRF7 activation of ISRE. The result (**Response Fig. 1A**) reveals that HBZ enhances IRF7-mediated ISRE activation in Jurkat, agreeing with our findings in HeLa (**Response Fig. 1B**). We also assessed the influence of HBZ on IRF3-driven ISRE activation and found that HBZ attenuates IRF3-dependent ISRE activation (**Response Fig. 1A and B**), consistent with the observations by Narulla et al.

We agree with you that the inhibitory effect of HBZ on IRF3 might counterbalance its enhanced effect on IRF7 activation, resulting in no overall effect on the IFN-I pathway. As shown below (**Response Fig. 1A and B**), while the co-expression of IRF3 and IRF7 increased ISRE activation, the introduction of HBZ resulted in a dose-dependent inhibition in both Jurkat and HeLa.

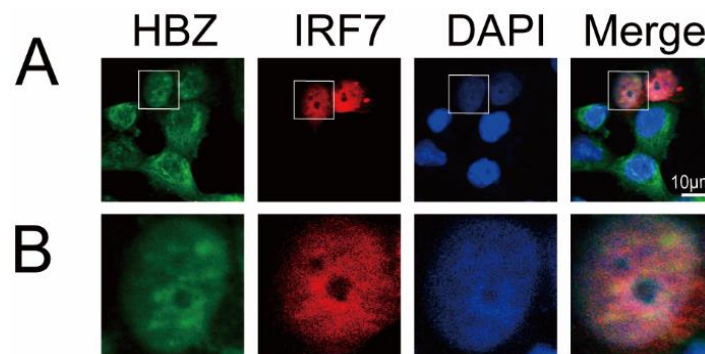


Response Fig. 1 (Fig. 4m and n in the revised version)

-HBZ displays a distinctive punctate localization in the nuclei of cells. This localization is not apparent in Figure 3B. It is also not clear whether IRF7 and HBZ colocalized in the nuclei of these cells. Can the authors address this?

Author Response:

Thank you for raising an important question concerning the subcellular localization pattern of HBZ. Now we have provided high-resolution images of the immunofluorescence staining result of HBZ (**Response Fig. 2A and B**), which indicates its punctate localization. Furthermore, these high-resolution images also demonstrate the colocalization of HBZ and IRF7 in the nuclei of cells.

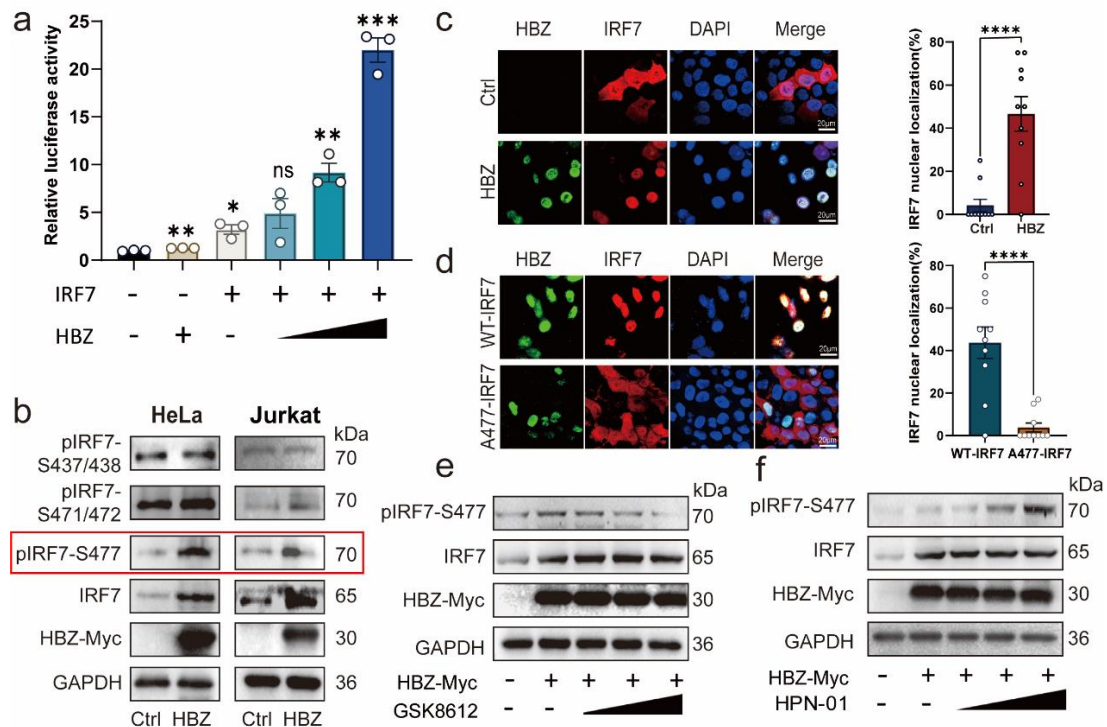


Response Fig. 2

-“C” needs to be added to this figure

Author Response:

We have reorganized the panels to improve the readability (**Response Fig. 3**). We apologize for this inconvenience.

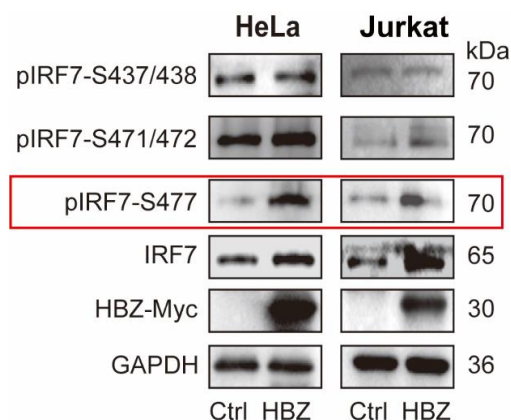


Response Fig. 3 (Fig. 3a-f in the revised version)

-Did the authors analyse phosphorylation of sites other than Ser477 in IRF7.

Author Response:

In the current study, our functional analyses focused on Ser477, given that this site has been identified as a principal activation site essential for IRF7 transcriptional activity and nuclear translocation (Lee et al., 2014; Lin, Mamane, & Hiscott, 2000; Ma, Huang, Wang, Wang, & Gao, 2023). Nevertheless, IRF7 does contain additional serine residues that have also been reported to promote its function, such as Ser437/438 and Ser471/472. To better answer your question, we performed Western blot analyses employing phospho-specific antibodies against different serine residues of IRF7. Consistent with our prior observations, Ser477 remains to be the most important phosphorylation site of IRF7 increased by HBZ (**Response Fig. 4**). These additional results confirm that Ser477 of IRF7 is the key phosphorylation site targeted by HBZ.



Response Fig. 4 (Fig. 3b in the revised version)

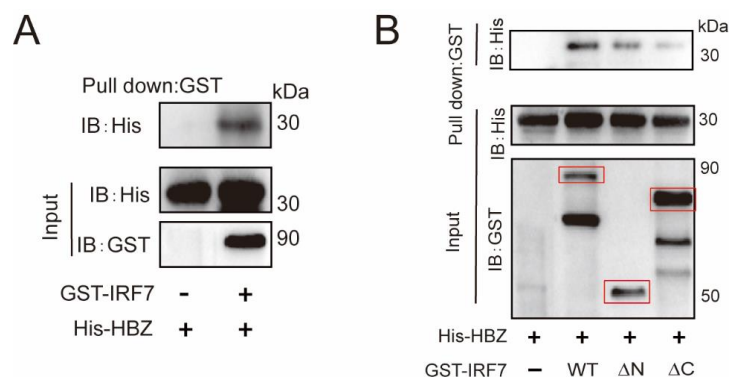
Lee, K. J., Ye, J. S., Choe, H., Nam, Y. R., Kim, N., Lee, U., & Joo, C. H. (2014). Serine cluster phosphorylation liberates the C-terminal helix of IFN regulatory factor 7 to bind histone acetyltransferase p300. *J Immunol*, 193(8), 4137–4148. doi:10.4049/jimmunol.1401290

Lin, R., Mamane, Y., & Hiscott, J. (2000). Multiple regulatory domains control IRF-7 activity in response to virus infection. *J Biol Chem*, 275(44), 34320–34327. doi:10.1074/jbc.M002814200

Ma, W., Huang, G., Wang, Z., Wang, L., & Gao, Q. (2023). IRF7: role and regulation in immunity and autoimmunity. *Front Immunol*, 14, 1236923. doi:10.3389/fimmu.2023.1236923

-Authors show that the binding of HBZ to IRF7 inhibits the binding of IRF7 to IRF3 suggesting that this may prevent the induction of IFN-I. Can the authors comment on a mechanism. Does HBZ bind the same domain as IRF3 on IRF7?

IRF3 and IRF7 are known to interact via their C-terminal domains ([Chen & Royer, 2010](#))([Sato et al., 2000](#)). According to our Co-IP result (**original Fig. 4F**), HBZ mainly targets the C-terminal domain of IRF7, similarly as IRF3 does. During revision, we further performed GST pull-down assays and confirmed the direct interaction between HBZ and IRF7 (**Response Fig. 5A**). In particular, we verified that HBZ also directly binds to the C-terminal region of IRF7 (**Response Fig. 5B**). Therefore, we assume that HBZ very likely competes with IRF3 to interact with IRF7 via its C-terminal region. We appreciate your insightful comment that inspired us to dive deep into the mechanism underlying the IFN-I inhibition by HBZ.



Chen, W., & Royer, W. E., Jr. (2010). Structural insights into interferon regulatory factor activation. *Cell Signal*, 22(6), 883–887. doi:10.1016/j.cellsig.2009.12.005

Sato, M., Suemori, H., Hata, N., Asagiri, M., Ogasawara, K., Nakao, K., . . . Taniguchi, T. (2000). Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN- α / β gene induction. *Immunity*, 13(4), 539–548. doi:10.1016/s1074-7613(00)00053-4

Reviewer #2 (Remarks to the Author):

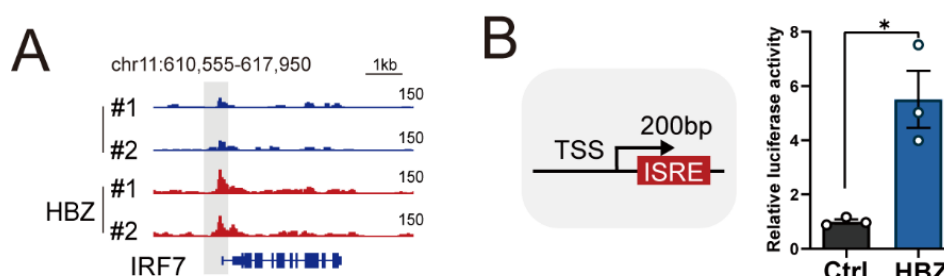
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Author Response:

We are grateful for your invaluable suggestions that helped us improve the quality of our manuscript. In Figure 2, the Authors clearly demonstrated that HBZ was enriched upstream of the TSS of IRF7. Authors should consider the use of the promoter (compare the wild-type with an IRF7 putative binding site mutant) reporter assay in HeLa, to further demonstrate that the “specificity” of such binding/enrichment is a major contributor to the IRF7 protein expression.

Author Response:

Thank you for your comment. As suggested, we cloned the promoter of IRF7 into a luciferase reporter and performed Luc assay in HeLa (**Response Fig. 6B**). As shown below, HBZ markedly enhanced luciferase activity driven by the wild-type IRF7 promoter, indicating that the enrichment of HBZ upstream of the locus of IRF7 (**Response Fig. 6A**) does increase its transcriptional activation (**Response Fig. 6B**).

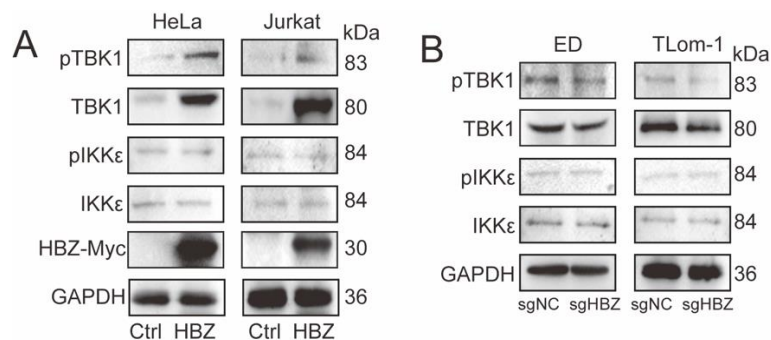


Response Fig. 6 (Fig. 2g and h in the revised version)

In Figure 3C, how does HBZ exactly promote IRF7 Ser477 phosphorylation? Is it due to the induction of TBK1 expression by HBZ as described in the manuscript, leading to more phosphorylation of IRF7? Was the induction of “excess” TBK1 necessary for the IRF7 activation (phosphorylation)? In a normal situation, the abundance of kinases is not the limiting factor. Protein expression of TBK1 can be included together with Figure 3G.

Author Response:

We fully agree that increased abundance of TBK1 by HBZ might not necessarily lead to the Ser477 phosphorylation of IRF7. Therefore, we further examined the effect of HBZ on the phosphorylation of TBK1, its active form. As shown below, HBZ not only increases the total amount of TBK1, but also promotes its phosphorylation (**Response Fig. 7A and B**). In contrast, neither the abundance nor the phosphorylation of IKK ϵ is increased by HBZ (**Response Fig. 7A and B**). As such, we propose that TBK1 phosphorylation as well as its upregulation by HBZ likely account for the downstream IRF7 activation. We greatly appreciate your inspiring comment.



Response Fig. 7 (Fig. 3g and h in the revised version)

In Figure 4D, the authors claim that HBZ binding to IRF7 inhibits IRF3-IRF7-mediated interferon signaling. First, authors can indicate whether ionic binding or hydrogen binding is predicted for HBZ-IRF7, including the distance between the predicted interaction and the overall binding energy, so that the authors have an idea of how strong the binding is predicted by AlphaFold.

Author Response:

We thank the reviewer for this important suggestion. We have now performed a detailed structural analysis of the HBZ-IRF7 complex using PISA (Protein Interfaces, Surfaces and Assemblies). The results indicate that both ionic (salt bridge) and hydrogen bonding interactions contribute significantly to the HBZ-IRF7 interface, with a predominant role for electrostatic interactions driving the high-affinity binding.

(1) Hydrogen bonding interactions

A total of 9 hydrogen bonds (**Response Table 1**) were identified at the HBZ-IRF7 interface, with distances ranging from 2.5–3.9 Å (mean: 3.2 Å).

Hydrogen Bonds				
HBZ Residue	Atom	Distance (Å)	IRF7	Atom
ARG 152	NH1	3.5	SER 484	O
ARG 152	NH1	3.7	SER 487	OG
ARG 152	NH2	3.5	SER 484	O
LYS 186	NZ	3.8	GLN 421	OE1
CYS 14	SG	3.3	ARG 398	NH1
GLU 21	OE1	2.5	TYR 377	OH
LEU 27	O	3.9	TYR 345	OH
ASP 188	OD2	2.7	THR 93	OG1
TYR 195	OH	2.9	ARG 49	NE

Response Table 1 (Supplementary Table 1 in the revised version)

(2) Ionic (salt bridge) interactions

A total of 26 salt bridges (**Response Table 2**) were identified, with distances ranging from 2.3–3.9 Å (mean: 3.1 Å).

Salt Bridges				
HBZ Residue	Atom	Distance (Å)	IRF7	Atom
HIS 149	NE2	2.9	ASP 491	OD1
HIS 149	NE2	3.9	ASP 491	OD2
ARG 152	NH1	3.2	ASP 491	OD2
LYS 153	NZ	3.8	GLU 498	OE1
LYS 153	NZ	3.3	GLU 498	OE2
GLU 16	OE1	2.4	LYS 373	NZ
GLU 16	OE2	3.2	LYS 373	NZ
GLU 16	OE2	2.9	ARG 398	NH1
GLU 16	OE2	3.8	ARG 398	NH2
ASP 17	OD1	2.7	ARG 370	NH1
ASP 17	OD1	3.7	ARG 370	NH2
ASP 17	OD2	3.3	ARG 370	NH1
ASP 17	OD2	2.8	ARG 370	NH2
GLU 21	OE1	2.7	LYS 375	NZ
GLU 21	OE2	2.3	LYS 375	NZ
GLU 31	OE1	3.1	LYS 296	NZ
GLU 31	OE2	3.9	LYS 296	NZ
GLU 185	OE2	3.9	ARG 418	NH2
GLU 187	OE1	3.6	ARG 100	NH2
GLU 187	OE1	2.3	ARG 100	NH1
GLU 187	OE2	3.5	ARG 100	NE
GLU 187	OE2	3.4	ARG 100	NH2
GLU 187	OE2	2.4	ARG 100	NH1
ASP 188	OD1	3.1	ARG 96	NE
ASP 188	OD1	2.8	ARG 96	NH1
ASP 188	OD1	2.6	ARG 96	NH2
GLU 187	OE2	2.4	ARG 100	NH1
ASP 188	OD1	3.1	ARG 96	NE
ASP 188	OD1	2.8	ARG 96	NH1
ASP 188	OD1	2.6	ARG 96	NH2

Response Table 2 (Supplementary Table 2 in the revised version)

(3) Interface summary

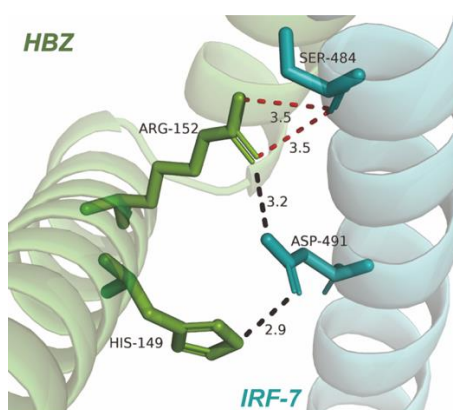
The HBZ-IRF7 interaction is predominantly driven by electrostatic forces (26 salt bridges, mean distance 3.1 Å), supplemented by hydrogen bonding (9 H-bonds, mean distance 3.2 Å). The estimated binding free energy of −35 kcal/mol predicts high-affinity binding in the nanomolar range (Response Table 3).

Interface Summary		
	HBZ	IRF7
Atoms in the interface	265 (15.4%)	271 (7.1%)
on the surface	1482 (86.2%)	2764 (72.3%)
total	1720 (100.0%)	3825 (100.0%)
idues in the interface	62 (30.1%)	80 (15.9%)
on the surface	206 (100.0%)	488 (97.0%)
total	206 (100.0%)	503 (100.0%)
Buried ASA, sq. Å	2750.1 (12.2%)	2617.6 (6.4%)
Total ASA, sq. Å	22495.6 (100.0%)	40875.7 (100.0%)
Solvation energy kcal/mol	-84.2	-343.9
SE gain, kcal/mol	-21.5	-13.6

Response Table 3 (Supplementary Table 3 in the revised version)

The figure is therefore replaced by an updated version and shown in our revised manuscript as **Fig. S4b**. The figure legend now reads as following:

"(b) Structural basis of HBZ-IRF7 interaction. Detailed view of intermolecular contacts, including hydrogen bond between HBZ ARG152 and IRF7 SER484 (3.5 Å), salt bridge between HBZ LYS153 and IRF7 GLU498 (3.3–3.8 Å), and salt bridge between HBZ HIS149 and IRF7 ASP491 (2.9–3.9 Å). The interface is predominantly driven by electrostatic interactions (26 salt bridges, mean 3.1 Å) supplemented by hydrogen bonding (9 H-bonds, mean 3.2 Å), with estimated binding free energy of −35 kcal/mol."

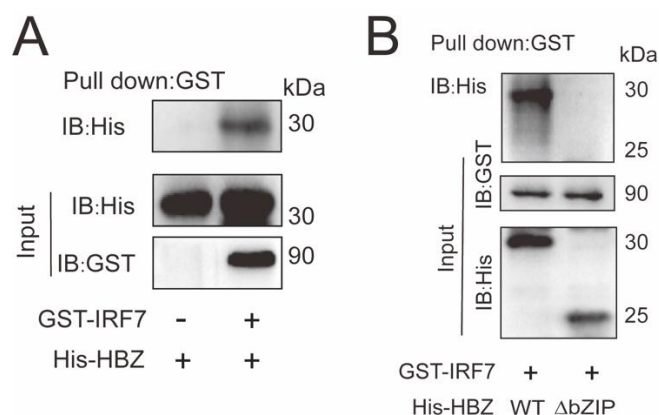


Response Fig. 8 (Supplementary Fig. 4b in the revised version)

In Figure 4E-G, the IP data showed the association of overexpressed IRF7 and HBZ, but not the direct interaction between them. Since this is the key hypothetical model of HBZ-mediated interferon antagonism, a direct interaction between IRF7 and HBZ is required. The use of purified recombinant proteins (including the bZIP mutant) for direct protein-protein interactions and binding affinity (Biacore, ITC, etc.) should be measured. The DNA contamination in the recombination protein should be excluded since the bZIP domain of HBZ is responsible for the binding.

Author Response:

We appreciate the importance of demonstrating the direct binding between HBZ and IRF7. To this end, we expressed and purified recombinant HA-HBZ and GST-IRF7 proteins. By performing a GST pull-down assay, we observed direct interaction between HBZ and IRF7 (**Response Fig. 9A**). Furthermore, a truncated HBZ protein without bZIP domain exhibited no binding, indicating that this domain is indispensable for the HBZ-IRF7 interaction (**Response Fig. 9B**). These in vitro findings provide compelling evidence for a direct interaction between HBZ and IRF7 mediated by the HBZ bZIP domain.



Response Fig. 9 (Fig. 4h and i in the revised version)

In Figure 4, the authors claim that HBZ interferes with the IRF3-IRF7 interaction to prevent IFN-I activation using a non-induced zebrafish model. Further improvement of data quality is needed.

Author Response:

We appreciate your suggestions that help us improve the clarity of this manuscript.

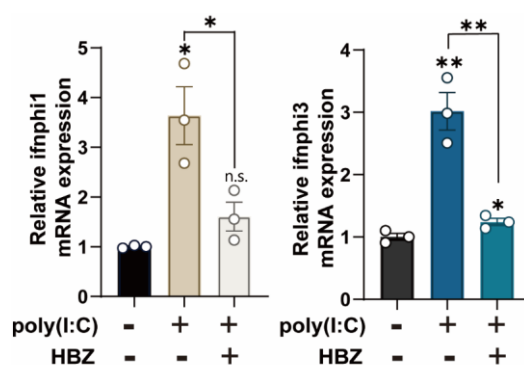
In Figure 4A, if no induction of the IFN pathway, or basal expression of IFN-I/ISG is low/absent in

normal zebrafish ESC, how can “IFN pathway was inactive in HBZ-expressing zebrafish ESC, line 179” be concluded?

Author Response:

This is a good point. What we intended to describe is, IRF7 overexpression normally would lead to IFN-I activation, but it did not in HBZ-expressing zebrafish ESC despite it is overexpressed because of HBZ. We admit that our prior description was unclear and might have caused misunderstanding. We apologize for this inconvenience. Now we have rephrased the text as follows, “However, by analyzing the gene set of the IFN-I pathway (KEGG NETWORK: N00150, Name: Type I IFN signaling pathway), we found representative IFN-I genes were not induced in HBZ-expressing zebrafish ESC despite IRF7 upregulation”.

On the other hand, to verify the inhibitory effect of HBZ on the IFN-I pathway and also to further address your concern, we have performed poly(I:C) stimulation to first activate the IFN-I pathway. Zebrafish ESCs expressing either HBZ or an empty control construct were treated with 10 µg/ml poly(I:C) for 24 hours. qPCR analysis demonstrates that the expression of representative type I interferons of zebrafish, *ifnφ1* and *ifnφ3* (Boudinot, Langevin, Secombes, & Levraud, 2016), was induced by poly(I:C) (Response Fig. 10), indicating activation of the IFN-I pathway. In contrast, the presence of HBZ significantly attenuated poly(I:C)-induced *ifnφ1* and *ifnφ3* expression, providing direct evidence of HBZ-mediated suppression of IFN-I induction (Response Fig. 10).



Response Fig. 10 (Fig. 4c in the revised version)

Reference

Boudinot, P., Langevin, C., Secombes, C. J., & Levraud, J. P. (2016). The Peculiar Characteristics of Fish Type I Interferons. *Viruses*, 8(11). doi:10.3390/v8110298

Referring to Figure 4B, “Analysis of the scRNA-seq data showed that the IFN-I pathway was not active either in ATLL clinical samples”. Only limited information was provided (line 180), and no guidance was given to help the reader interpret the scRNA-seq data.

Author Response:

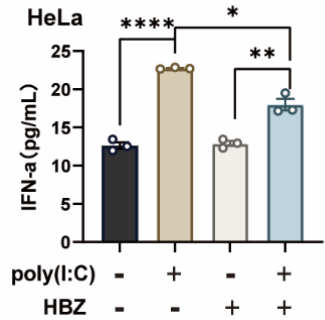
We apologize for the insufficient description. scRNA-seq data analysis was performed by NovelBio Co.,Ltd. with NovelBrain Cloud Analysis Platform. Datasets of ATLL patients and healthy controls were obtained from the ENA database (PRJEB47382). We applied fastq with default parameter filtering the adaptor sequence and removed the low-quality reads to achieve the clean data. UMI-

tools was applied for Single Cell Transcriptome Analysis to identify the cell barcode whitelist. The UMI-based clean data was mapped to human genome (Ensemble version 91) utilizing STAR mapping with customized parameter from UMI-tools standard pipeline to obtain the UMIs counts of each sample. To identify differentially expressed genes among samples, the function FindMarkers with wilcox rank sum test algorithm was used under following criteria: 1. logFC > 0.25; 2. pvalue<0.05; 3. min.pct>0.1. Based on the KEGG database (KEGG NETWORK: N00150, Name: Type I IFN signaling pathway), we conducted heatmap imaging for the key IFN-I genes. The above description was added in the Materials and Methods of our revised manuscript.

In Figure 4H, IFN should be activated so that the HBZ effect can be observed.

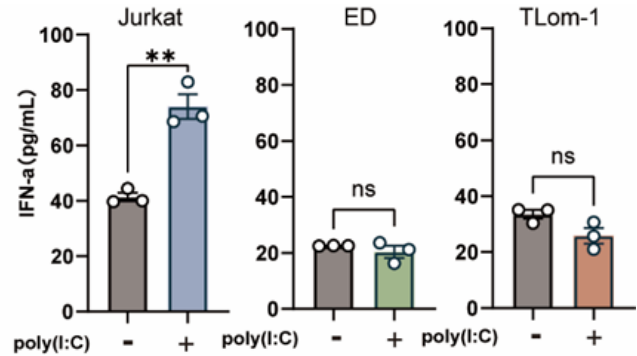
Author Response:

We agree that it only makes sense to conclude HBZ inhibits IFN when the pathway is activated in the first place. As such, we treated the cells with poly(I:C) to activate the type I IFN pathway and then quantified IFN-α production by ELISA (**Response Fig. 11**). Consistent with our hypothesis, HBZ expression substantially diminishes IFN-α induction (**Response Fig. 11, column 4 vs 2**).



Response Fig. 11 (Fig. 4c in the revised version)

However, since poly(I:C) could not active IFN-I in ED or TL-om1 (**Response Fig. 12**), it is difficult to conclude whether the inactive IFN-I pathway in these cell lines is because of HBZ or not. As such, we have removed the **original Fig. 4H** for better clarity in the revised manuscript.



Response Fig. 12

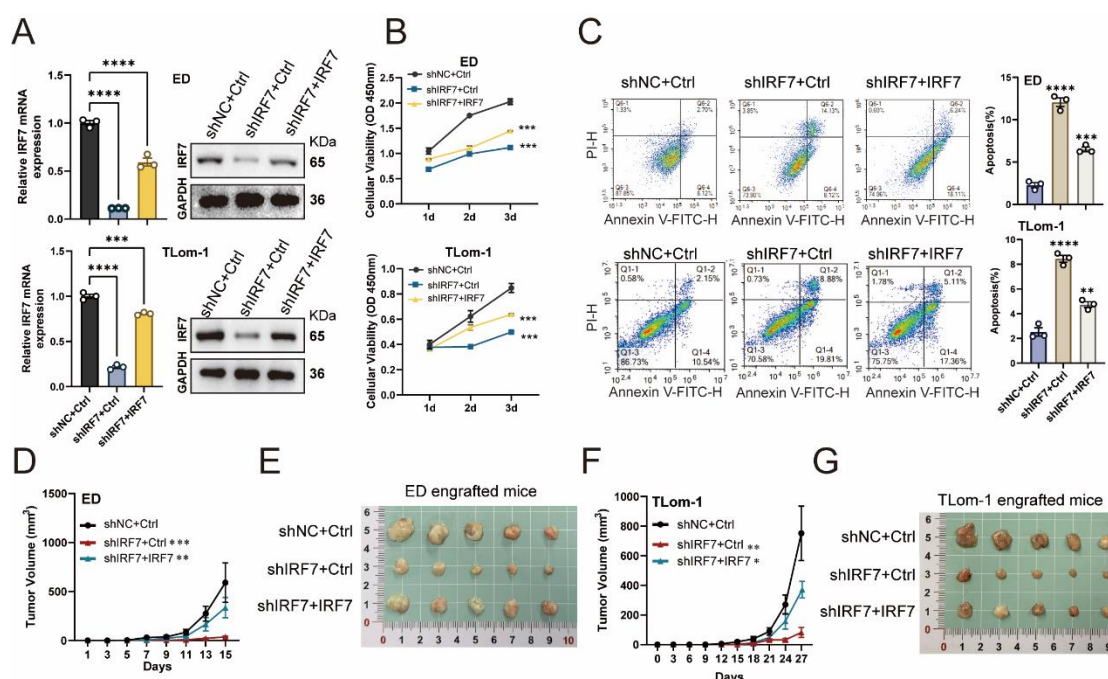
In Figure 5, how the “ATLL cells stably expressing IRF7-targeting shRNA” be generated, if the

IRF7 knockdown greatly impaired cell proliferation (Fig. 5B) and increased apoptosis (Fig. 5C) in ATLL cell lines. It is recommended to include a control: an expression plasmid for IRF7 with the shRNA targeting sequence mutated. This control should be added in the experiments described in Figure 5.

Author Response:

Since xenograft experiments required large number of cells, instead of using siRNA, we performed IRF7 shRNA in ATLL cell lines by lentiviral transduction. We did not select stable lines so that our prior description “ATLL cells stably expressing IRF7-targeting shRNA” was inaccurate. Thank you very much for pointing it out. We sincerely apologize for this error and any inconvenience it may have caused. Now we have rephrased the sentence as the following, “Next, we established xenograft tumor models by subcutaneous injection of ATLL cells expressing IRF7-targeting shRNA into immunodeficient mice.”

As suggested, we included a control to examine the rescue effect of IRF7. Initially, we tried to follow your suggestion exactly by introducing mutations in the IRF7 expression plasmid to avoid being targeted by shRNA. However, since the newly introduced mutations in IRF7 affected its function, we decided to use UTR-targeting shRNAs. Unfortunately, although we tested multiple new shRNAs targeting distinct regions of IRF7 UTR, none of which yielded ideal knockdown efficiency. As a result, we had to switch back and use the original shRNA and performed the rescue experiment by simply overexpressing the wild-type IRF7. Nevertheless, this method worked and our new results confirm that IRF7 indeed plays a vital role in ATLL cell survival: partially restoring IRF7 expression attenuated apoptosis *in vitro* and restored tumorigenic capacity *in vivo*, thereby suggesting that the observed phenotypes were specifically due to IRF7 silencing rather than off-target effects (Response Fig. 13).



Response Fig. 13 (Fig. 5a-g in the revised version)

Reviewer #3 (Remarks to the Author):

In this study, Yuan et al. reported on the activation of IRF7 by HTLV-1 oncoprotein HBZ. The findings are of great interest as IRF7 was initially discovered as an oncoprotein activated by EBV oncoprotein LMP1. Multiple lines of evidence have been presented to make the point. However, the paper could be revised to focus more on the key finding. Data such as Figure 1 might be moved to supplementary. Several mechanisms for HBZ perturbation of IRF7 have been suggested. This is not surprising since viral oncoproteins are commonly very pleiotropic, but it will still be helpful if one key mechanism is characterized more thoroughly. Generally, the novel and interesting findings substantially advance the field.

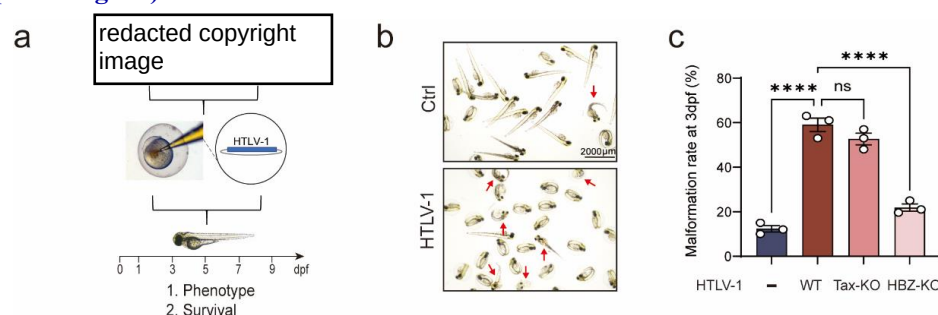
Author Response:

Thank you very much for acknowledging our work.

1) Figures 1B and 1C: It appears that the same data were used twice. The two figures should be combined.

Author Response:

Thank you for pointing out this error. We have now combined these two figures as suggested (Response Fig. 14).

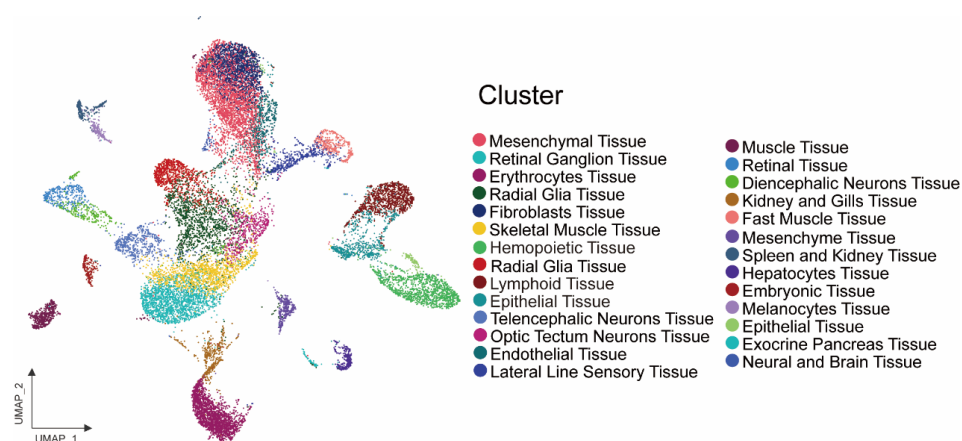


Response Fig. 14 (Fig. 1a-c in the revised version)

2) Figures 2A and 2B: Please specify the scRNA-seq cluster names.

Author Response:

We apologize for missing this information. The 27 distinct clusters are now named and annotated based on integrated marker gene expression and canonical zebrafish subtypes (Response Fig. 15) (Farnsworth, Saunders, & Miller, 2020).



Response Fig. 15 (Fig. 2a in the revised version)

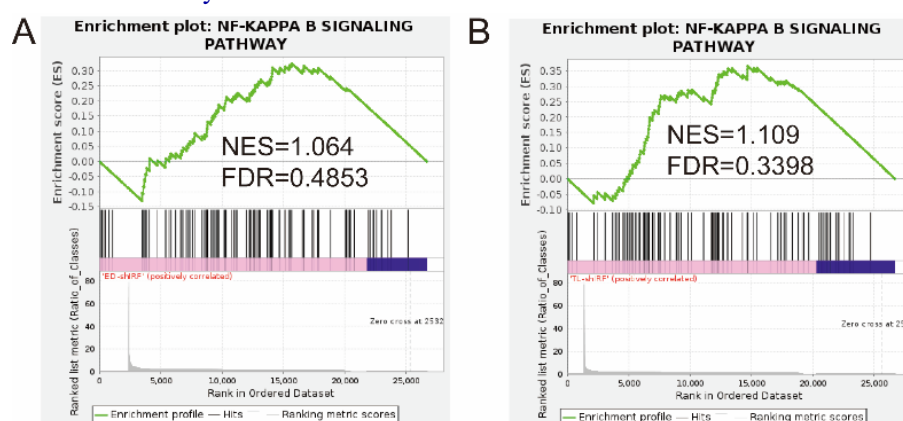
Reference

Farnsworth, D. R., Saunders, L. M., & Miller, A. C. (2020). A single-cell transcriptome atlas for zebrafish development. *Dev Biol*, 459(2), 100–108. doi:10.1016/j.ydbio.2019.11.008

3) Figures 2D and 4H: Does IRF7 drive NF- κ B activation?

Author Response:

Thank you for raising an interesting question regarding potential crosstalk between IRF7 and NF- κ B pathways in the context of HBZ function. To address this point, we performed further analyses on the relevant RNA-seq datasets. As shown below (**Response Fig. 16**), the NF κ B pathway does not seem to be activated by IRF7.



Response Fig. 16

4) Figure 2J: Can CRISPR knockout influence mRNA levels?

Author Response:

Thank you for this important question. In principle, CRISPR/Cas9 creates a double-strand break (DSB) in the DNA, which will be repaired by the cell via non-homologous end joining (NHEJ), thereby introducing small insertions or deletions (indels) into the genome. If the indel disrupts the reading frame, a premature termination codon (PTC) could be created. When mRNAs contain a PTC >50-55 nucleotides upstream of an exon-exon junction, they are usually flagged for degradation by the nonsense-mediated mRNA decay (NMD) pathway that leads to their degradation (Popp & Maquat, 2016). Therefore, it is common to see a decrease in the mRNA level of the target gene when performing CRISPR/Cas9 knockouts, as what we saw in the **original Fig. 2J**.

Reference

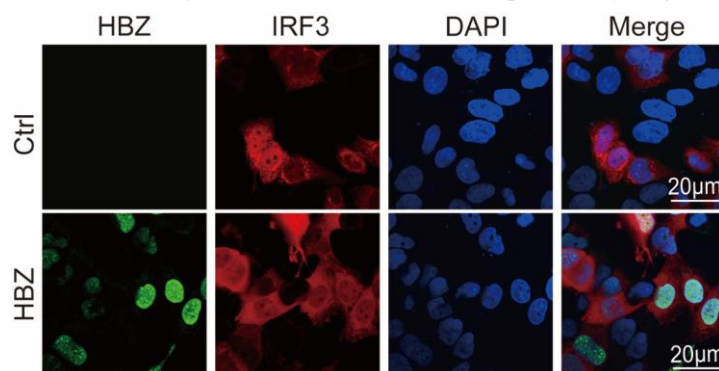
Popp, M. W., & Maquat, L. E. (2016). Leveraging Rules of Nonsense-Mediated mRNA Decay for Genome Engineering and Personalized Medicine. *Cell*, 165(6), 1319–1322. doi:10.1016/j.cell.2016.05.053

5) Figure 3: It will be of interest to verify whether HBZ alters IRF3 nuclear translocation.

Author Response:

Thank you for this valuable suggestion. To address this point, we performed immunofluorescence experiment in HeLa co-expressing HBZ and IRF3. Our analysis showed that IRF3 exhibits a mixed localization pattern with both nuclear and cytoplasmic proportion present. In the presence of HBZ,

this mixed pattern did not change, or at least HBZ did not promote the nuclear translocation of IRF3 (Response Fig. 17). These findings further confirm that HBZ specifically targets IRF7 but not IRF3.

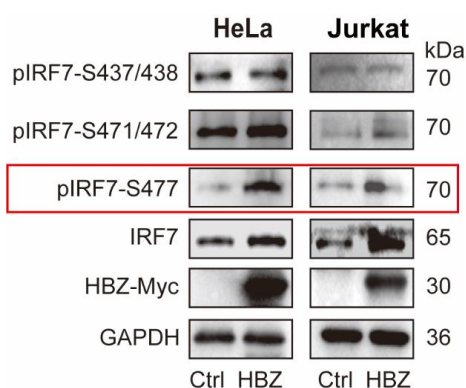


Response Fig. 17 (Supplementary Fig. 4a in the revised version)

6) Figure 3D: The observed increase in phosphorylated IRF7 (pIRF7) could simply reflect higher IRF7 levels.

Author Response:

Thank you for raising this critical point. As shown below, our additional results indicate although HBZ upregulates IRF7 expression, it only markedly increases Ser477 phosphorylation of IRF7 but not Ser437/438 or Ser471/472. Therefore, the observed increase in phosphorylated IRF7 in **original Fig. 3D** appears to be a result of IRF7 activation by HBZ rather than resulting from an increase in total IRF7 abundance.

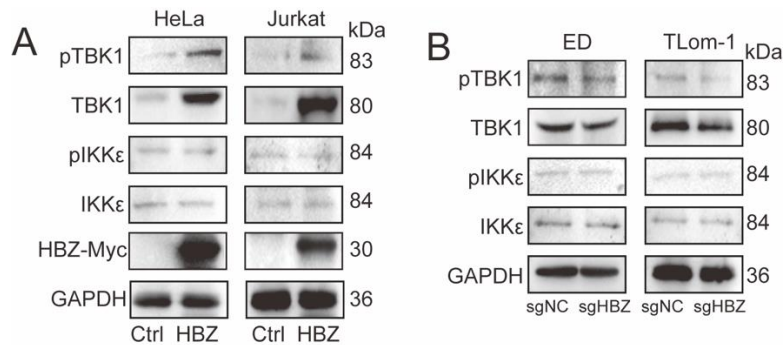


Response Fig. 18 (Fig. 3b in the revised version)

7) Figure 3I: TBK1 detection should be confirmed by Western blotting. The result is weak.

Author Response:

Thank you for your comment. As suggested, WB analysis of TBK1 was performed and shown below. The WB result is consistent with our qPCR results.

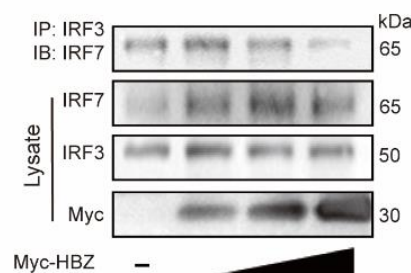


Response Fig. 19 (Fig. 3g and h in the revised version)

8) Figure 4G: Immunoprecipitation of endogenous proteins might provide more physiologically relevant insights.

Author Response:

Based on your suggestion, we examined the interaction of endogenous IRF3 and IRF7 in HeLa in the presence of HBZ. As shown below, the result agrees with our overexpression model and suggests HBZ dose-dependently suppresses IRF3-IRF7 interaction (**Response Fig. 20**).



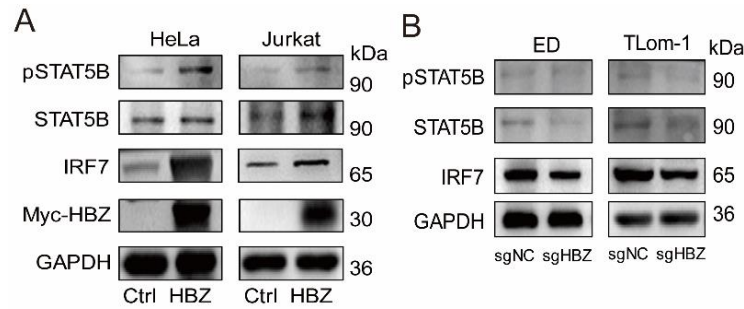
Response Fig. 20 (Fig. 4I in the revised version)

9) Does HBZ activate STAT5B? Additionally, is STAT5B activation observed in HTLV-1-infected cells?

Author Response:

To investigate this, we overexpressed HBZ in HeLa and Jurkat and examined the phosphorylation of STAT5B. Our results showed that HBZ enhanced IRF7 as well as STAT5B phosphorylation, suggesting the potential STAT5B activation by HBZ (**Response Fig. 21A**). Furthermore, we confirmed that STAT5B could also be activated by endogenous HBZ in ATLL cell lines (**Response Fig. 21B**).

Our main conclusion is that HBZ/IRF7 promote STAT5B expression. Although we have shown here that STAT5B activation (phosphorylation) might also be promoted, since the pSTAT5B is very difficult to detect (**Response Fig. 21B**), we are uncertain whether STAT5B is widely activated in HTLV-1 infected cells. We apologize for this technical difficulty.

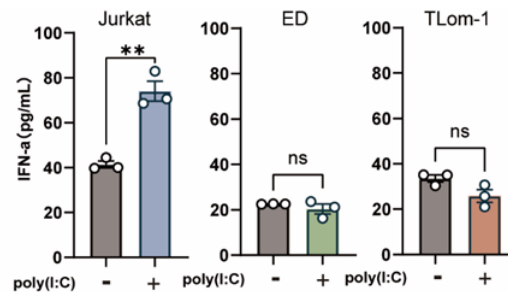


Response Fig. 21 (Fig. 6j and k in the revised version)

10) Lines 189-90: “It seems the activation of IRF7 in HTLV-1 infected cells did not necessarily lead to the activation of IFN-I, probably owing to the presence of HBZ.” More experiments should be carried out to test it out. Integrity of IFN-I production pathway in the cell line should be verified by transfecting RNA mimic poly (I:C) or DNA mimic VACV70 mer to activate RNA or DNA sensing pathway. Moreover, they should test if HBZ suppresses IFN production pathway by comparing IFN-I levels in HBZ-overexpressing cell with activated IFN production pathway.

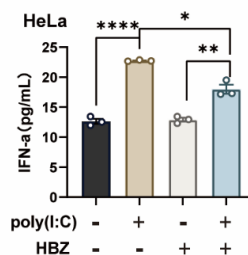
Author Response:

We appreciate these critical questions. We have measured IFN- α production in Jurkat, ED and TLom-1 cells by ELISA. As shown below, all the cell lines produced IFN- α , suggesting that there is no intrinsic deficiency in IFN- α expression (**Response Fig. 22**). However, although poly(I:C) stimulation increased IFN- α production in Jurkat, it failed to do so in HTLV-1 infected cells (**Response Fig. 22**). We understand that our previous statement was inaccurate, therefore we have remove it in our revised manuscript.



Response Fig. 22

On the other hand, while poly(I:C) stimulation increased IFN- α production in HeLa, when HBZ was overexpressed IFN- α production was markedly suppressed, confirming its inhibitory role in IFN-I (**Response Fig. 23, column 4 vs 2**).



Response Fig. 23 (Fig. 4c in the revised version)