EMBO reports

High salt activates p97 to reduce host antiviral immunity by restricting Viperin induction

Yukang Yuan, Ying Miao, Tengfei Ren, Fan Huang, Liping Qian, Xiangjie Chen, Yibo Zuo, Hong-Guang Zhang, Jiuyi He, Caixia Qiao, Qian Du, Qiuyu Wu, Wei Zhang, Chuanwu Zhu, Yang Xu, Depei Wu, Weifeng Shi, Jingting Jiang, Guoqiang Xu, and Hui Zheng
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Corresponding author(s): Hui Zheng (huizheng@suda.edu.cn)

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Dr. Zheng,

Thank you for the submission of your research manuscript to EMBO reports. We 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 email.

As you will see, the referees think 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 their points need to be addressed, I will not detail them here.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript or in the 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. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic, and we have therefore extended our 'scooping protection policy' to cover the period required for full revision. Please contact me to discuss the revision should you need additional time, and also if you see a paper with related content published elsewhere.

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:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

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

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

For more details, please refer to our guide to authors:
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See also our guide for figure preparation:
http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines:
http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

5) 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 a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

See also: http://embor.embopress.org/authorguide#datadeposition

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.

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 strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

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, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

9) Please also note our new reference format:
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10) For microscopic images, please add scale bars of similar style and thickness to all the microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend.

11) Please add up to five keywords to the title page.

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.

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

Referee #1:

In this work, Yuan and co-authors demonstrate that conditions of dietary high salt undermine the innate anti-viral immune responses against viral infection. Upon delineating the mechanisms underlying this phenomenon, authors demonstrated that high salt acts upon VCP/p97 ATPase, which becomes acetylated at K663 and promotes degradation of USP33 deubiquitinase. The lack of USP33 then leads to hyper-ubiqutination and degradation of viperin, which otherwise would have elicited its anti-viral effects. Reduction of salt in the diet reverses these phenotypes. Authors conclude on the importance of balancing salt amounts in the diet for anti-viral defenses.

This is an interesting and comprehensive work that contributes a substantial advance to our knowledge in regard to the modes of regulation of viperin production and anti-viral defenses. In addition to the mechanistic importance, this work is relevant to the importance of balancing salt in the diet for reducing the susceptibility to viral infections. The experiments are well designed and utilized multiple overlapping and complementary experimental models. The biochemical tour de force yielded exciting data, the importance of which was then tested in the in vivo viral infection models.

The results are very clear, and the conclusions are supported by compelling data. This work will be of interest for the readers of the EMBO Reports. Addressing the following minor points may further strengthen this work:
1. Even a minimal introduction of p97 and USP33 functions in the Abstract will help the readers with understanding the impact of presented studies.
2. In description of experiments shown in Fig 1A, authors may want to point out that even prolonged diet increases viral load - it is just that this increase does not reach significance - unlike short term salt diet.
3. Description of experiment shown in Fig 2 lacks clarity. Authors should just state that already high viral load in Ifnar1-null or Stat1-null cells was not further increased by high salt.
4. Use of differentially colored lines for NSD versus HSD conditions will improve the perception of Fig. 2N and 4I.
5. Line 251. Consider replacing "damaging viperin stability" with "decreasing viperin stability". In general, some attention of a good English editor could improve the readability of this work.
6. Line 390: renin, not rennin

Referee #2:

The author's report some interesting observations on the effects of a high salt diet on the ability of mice to fight off viral infections. These observations are followed up by studies in cell lines and lead the authors to the conclusion that high salt indirectly leads to the down-regulation of viperin through a process that involves acetylation of p97 and the depletion of the deubiquitinase (DUB) USP33.

The most interesting aspect of the paper (to this reviewer) is insight into the regulation of viperin expression by the DUB USP33. It is not clear however, how this chain of regulation is biochemically transmitted from the initial signal i.e. high NaCl. Furthermore it would seem that many proteins would be affected (the immune system has many components) and it is not clear how the authors draw the line from salt to p97 and then to USP33 and viperin. Further explanation would be helpful.

The paper reports a very large amount of data in a rather condensed form - figures with multiple panels that can be hard to follow. I think this work would be better presented as a full-length paper rather than a 'report' format.

In particular, proteomic analysis plays a key role in identifying viperin as a target of high salt. However, the details of this experiment are not presented - the expression of other proteins would certainly have been found to be salt sensitive. Details of the data analysis need to be presented, even if only in the SI.

Referee #3:

In this manuscript, Yuan Y et al. found that the short duration treatment of high salt impaired host anti-viral capacity through P97 acetylation-promoted degradation of Viperin protein in macrophages and other types of cells. Furthermore, the study showed that low salt diet inhibited VSV infection in mice. Overall, the results in this study are interesting and solid, and also support its conclusions quite well.

Critique:
1, Addition of NaCl not only increases the sodium concentration in the media, but also upregulates the extracellular osmolarity. Then what is the main contributor to the impairment of anti-viral capacity? If extracellular sodium is more important, why does not its upregulation affect the viral infection after the long duration of treatment (Figure 1A and 1E).
2, Do type 1 interferon and viral infection regulate P97 acetylation?
3, Aldosterone-mineralocorticoid receptor system serves as the major regulator of fluid homeostasis in vivo and is readily activated by the treatment of low salt diet in mice. The authors need to address whether aldosterone-mineralocorticoid receptor

is important for the enhancement of anti-viral capacity by low-salt diet in mice.
4, Is it possible that viral infection may change the local concentration of extracellular sodium and the extracellular osmolarity in the infected tissue?
5, NaCl affects the universal ubiquitination in macrophages (Figure 5H-5J). What is the consequence? Does it affect any specific cellular physiology?

Referee #1:

In this work, Yuan and co-authors demonstrate that conditions of dietary high salt undermine the innate anti-viral immune responses against viral infection. Upon delineating the mechanisms underlying this phenomenon, authors demonstrated that high salt acts upon VCP/p97 ATPase, which becomes acetylated at K663 and promotes degradation of USP33 deubiquitinase. The lack of USP33 then leads to hyper-ubiqutination and degradation of viperin, which otherwise would have elicited its anti-viral effects. Reduction of salt in the diet reverses these phenotypes. Authors conclude on the importance of balancing salt amounts in the diet for anti-viral defenses.

This is an interesting and comprehensive work that contributes a substantial advance to our knowledge in regard to the modes of regulation of viperin production and anti-viral defenses. In addition to the mechanistic importance, this work is relevant to the importance of balancing salt in the diet for reducing the susceptibility to viral infections. The experiments are well designed and utilized multiple overlapping and complementary experimental models. The biochemical tour de force yielded exciting data, the importance of which was then tested in the in vivo viral infection models.

Thank you so much for these good comments!

The results are very clear, and the conclusions are supported by compelling data. This work will be of interest for the readers of the EMBO Reports. Addressing the following minor points may further strengthen this work:
1. Even a minimal introduction of p97 and USP33 functions in the Abstract will help the readers with understanding the impact of presented studies.

Done. Please see the corresponding introduction in Line 79-92.

2. In description of experiments shown in Fig 1A, authors may want to point out that even prolonged diet increases viral load - it is just that this increase does not reach significance - unlike short term salt diet.

Thanks! We added the description to point out this phenomenon. Please see it in Line 114-118. We also attached it here for your convenience:

"we noticed that as compared with an NSD, a continuous 30-day HSD seemed to slightly upregulate virus titers and viral RNA levels in the blood and spleens of mice challenged with viruses, but the difference was not significant (Figs 1A, EV1A and

EV1B)”

3. Description of experiment shown in Fig 2 lacks clarity. Authors should just state that already high viral load in Ifnar1-null or Stat1-null cells was not further increased by high salt.

Thanks! We corrected this description of this experiment. Please see it in new Line 168-170 and Line 182-183.

4. Use of differentially colored lines for NSD versus HSD conditions will improve the perception of Fig. 2N and 4I.

Done. Please see the new Fig. 2N and 4I.

5. Line 251. Consider replacing "damaging viperin stability" with "decreasing viperin stability". In general, some attention of a good English editor could improve the readability of this work.

Thank you for pointing out the inaccurate expression. We have corrected it. Please see it in new Line 277 and Line 1066. In addition, the manuscript has been edited by the American Journal Experts (AJE) (the verification code: 4A1D-B80D-C1DE-F031-F15A). Thanks!

6. Line 390: renin, not rennin

Thanks! It has been corrected (please see new Line 433**).**

Referee #2:

The author's report some interesting observations on the effects of a high salt diet on the ability of mice to fight off viral infections. These observations are followed up by studies in cell lines and lead the authors to the conclusion that high salt indirectly leads to the down-regulation of viperin through a process that involves acetylation of p97 and the depletion of the deubiquitinase (DUB) USP33.

The most interesting aspect of the paper (to this reviewer) is insight into the regulation of viperin expression by the DUB USP33. It is not clear however, how this chain of regulation is biochemically transmitted from the initial signal i.e. high NaCl. Furthermore it would seem that many proteins would be affected (the immune system

has many components) and it is not clear how the authors draw the line from salt to p97 and then to USP33 and viperin. Further explanation would be helpful.

Thanks for your considering our work as interesting observations, particularly in revealing the deubiquitinase USP33-mediated regulation of Viperin expression.

We are sorry that we did not clearly describe the chain of regulation in the old version of our manuscript. Thus, we added further explanation in the first paragraph of the Discussion section to help to understand the signaling transmission from high NaCl (please see Line 404-413**). We also attached it here for your convenience.**

"On the whole, high salt stimulates acetylation of p97 at Lys663, which activates p97 to induce degradation of certain ubiquitinated proteins. USP33, whose levels are critically controlled by p97-mediated degradation (*J Biol Chem. 2014 Jul 11;289(28):19789-98*), thus undertook a substantial decrease in protein levels under treatment of high salt. USP33 is an important deubiquitinase of antiviral protein Viperin and therefore its deficiency results in increased ubiquitination of Viperin protein and decreased protein levels of Viperin. As a potent antiviral protein induced by IFN-I signaling, Viperin deficiency in cells largely attenuates IFN-I antiviral activity upon viral infection, which consequently leads to a promotion of viral infection by high salt. "

We agree with the reviewer that there are other immune components, which could contribute to high salt-mediated inhibition of antiviral innate immunity. In our study, we demonstrated that high salt-activated p97 promotes USP33 downregulation, which in turn lowers antiviral Viperin protein levels, thus resulting in attenuated antiviral immunity. To draw the regulation line, we utilized gene knockout strategies, as well as other strategies, to make sure the key regulatory signaling proteins in each step of signaling transmission. We briefly described it as follows:

(1) By using IFN-I receptor-KO (*Ifnar1*$^{-/-}$) and STAT1-deficient (U3A) cells, we first confirmed that high salt-mediated inhibition of antiviral immunity is dependent on IFN-I signaling (please see Fig. 2A and 2D**). (2) Furthermore, by quantitative proteomic analysis, we found that Viperin, which is an IFN-I-induced potent antiviral ISG protein, is the most dramatically downregulated ISG protein by high salt. Importantly, by utilizing Viperin-KO (*Rsad2*$^{-/-}$) cells, we confirmed that high salt-mediated inhibition of antiviral immunity is dependent on Viperin (**Fig. 2L**). (3) Then, by analyzing Viperin mRNA**

levels and ubiquitination regulation using the pan-deubiquitinase inhibitor (PR-619), we further demonstrated that Viperin downregulation by high salt is dependent on the deubiquitinase activity (Fig. 3A and 3E). After identifying USP33 as the responsible deubiquitinase of Viperin, we used the *Usp33*$^{-/-}$ cells and confirmed that high salt-mediated inhibition of antiviral immunity is dependent on USP33 (Fig. 4F and Fig. 4H). (4) Given that we demonstrated that high salt regulates the levels of ubiquitinated-USP33 but not non-ubiquitinated-USP33 (Fig. 5A-5D), we speculated the possible role of p97, which is recognized as a common key protein to specifically recruit ubiquitinated proteins for degradation. Next, we further confirmed that the effects of high salt on USP33 levels, Viperin levels and antiviral immunity are all dependent on p97 by utilizing *p97*$^{-/-}$ cells, p97-knockdown cells and the specific p97 inhibitor (Fig. 5E, 5F, 5K, 5M, 5N and Fig. EV5I, EV5J). (5) We also confirmed the *in vivo* roles of both Viperin and USP33 in contributing to high salt-mediated inhibition of antiviral immunity by utilizing Viperin-KO *(Rsad2*$^{-/-}$*)* and *Usp33*$^{-/-}$ mice (Fig. 2N and Fig. 4I).

Based on these observations, we think that the NaCl-p97-USP33-Viperin pathway is the major line to contribute to high salt-mediated inhibition of antiviral immunity. Although there could be other immune components that contribute to high salt-mediated inhibition of antiviral immunity, we speculate that their effects could be weak or be largely compromised by some opposite signaling regulation.

The paper reports a very large amount of data in a rather condensed form - figures with multiple panels that can be hard to follow. I think this work would be better presented as a full-length paper rather than a 'report' format.

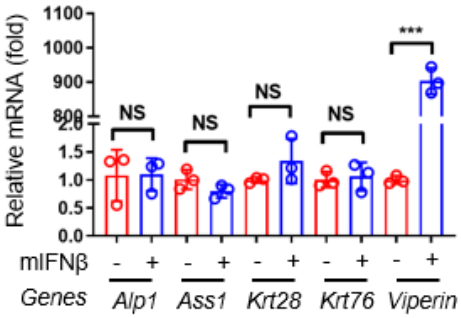
Thanks for pointing out a very large amount of data in this work. We make every effort to reveal the detailed mechanisms as possibly as we can. In addition, this work has actually been presented as a full-length paper for EMBO Reports.

In particular, proteomic analysis plays a key role in identifying viperin as a target of high salt. However, the details of this experiment are not presented - the expression of other proteins would certainly have been found to be salt sensitive. Details of the data analysis need to be presented, even if only in the SI.

Thanks for this comment! We added more details of this experiment in both the full text (please see new Line 198-211) and its legend (Line 994-1000).

In addition, we agree with the reviewer that other proteins could also be affected by high salt, as shown in our proteomic analysis data. By analyzing the proteomic data, we noticed that the changes of most of identified proteins are not very significant (less than 2 folds) under this condition of our high-salt experiment. However, we noticed five proteins (three downregulated proteins: Alp1, Rsad2, ASS1; two upregulated proteins: Krt28, Krt76) with significantly differential expression levels (downregulated proteins: the average ratio of high-salt to control in two repeated experiments is less than 0.5; upregulated proteins: the average ratio of high-salt to control is more than 2.0). Given that we have demonstrated by a series of experiments that high salt-mediated inhibition of antiviral immunity is dependent on IFN-I, we analyzed by the Interferome database (http://www.interferome.org/interferome/search/showSearch.jspx) whether these five changed proteins are IFN-I-associated gene products. Obviously, Rsad2 (Viperin) is an IFN-I-induced ISG that possesses potent antiviral activity. However, the other four proteins seem not to be IFN-I-induced gene products. Thus, in the old version, we focused on Viperin to analyze the effect of high salt. More importantly, by utilizing Viperin-KO *(Rsad2$^{-/-}$)* cell line and mice, we clearly demonstrated from *in vitro* and *in vivo* that Viperin is the main contributor to high salt-mediated inhibition of antiviral immunity.

Here, thanks for the reviewer's good comment. Thus, we further performed a new experiment to analyze whether these five significantly changed proteins are IFN-I-induced gene products. The results showed that only Viperin but not the other four proteins can be induced by IFN-I (please see Fig. EV3A, right). We also attached it here for your convenience:



Legend: *RT-qPCR was used to analyze mRNA levels of several top differential genes in RAW264.7 cells treated with mIFNβ (500 IU/ml) for 12 hrs.*

Collectively, we think that IFN-I-induced Viperin protein is the main contributor to high salt-mediated inhibition of antiviral immunity, although other proteins could contribute to some extent. In addition, according to the reviewer's suggestion, we also added the corresponding descriptions in the

new version of our manuscript (please see Line 202-211**).**

Referee #3:

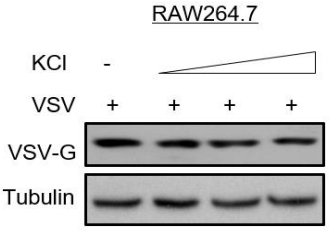
In this manuscript, Yuan Y et al. found that the short duration treatment of high salt impaired host anti-viral capacity through P97 acetylation-promoted degradation of Viperin protein in macrophages and other types of cells. Furthermore, the study showed that low salt diet inhibited VSV infection in mice. Overall, the results in this study are interesting and solid, and also support its conclusions quite well.

Thanks for your considering that our study is interesting and solid, and supports the conclusions quite well.

Critique:

1, Addition of NaCl not only increases the sodium concentration in the media, but also upregulates the extracellular osmolarity. Then what is the main contributor to the impairment of anti-viral capacity? If extracellular sodium is more important, why does not its upregulation affect the viral infection after the long duration of treatment (Figure 1A and 1E).

This is a very interesting question. To be honest, we actually do not know how high salt activates acetylation of p97, which finally impairs antiviral capacity. To make effort to provide more evidence, we further utilized the same concentrations of KCl as NaCl used in our study to get the same extracellular osmolarity. Interestingly, we noticed that unlike NaCl, the same concentrations of KCl did not significantly affect viral infection (please see the data below**), suggesting that the extracellular osmolarity is not the main contributor to the impairment of antiviral capacity. Here, we are not sure whether extracellular sodium is more important, since the extracellular chloride ion could also synergistically contribute to this response, which we think is an interesting new project and needs great numbers of experiments to address.**

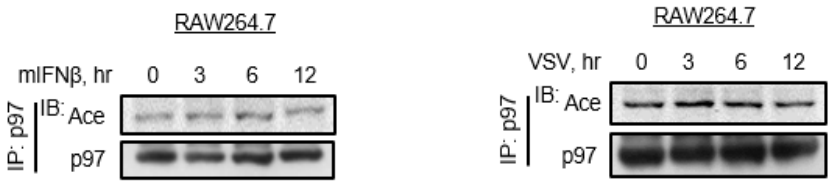


***Legend**: Western blot analysis of VSV-G levels in RAW264.7 cells infected with VSV (MOI=1.0) for 12 hrs immediately after addition of KCl (+17, 34 and 51 mM).*

As to Fig.1A and 1E，we would like to point out the changes affected by high salt. In Fig. 1A, we can actually see the tendency toward the increase in viral infection mediated by high salt. However, the increase is not significant. Based on our evidence in mouse *in vivo* experiments (Fig. 4C) and other analysis, we speculated that high levels of NaCl could be balanced and adapted during long-time treatment by many *in vivo* systems, such as arterial pressure, renal sympathetic tone and the renin-angiotensin-aldosterone system, which results in the attenuation of high salt-induced signaling activation *in vivo*. In Fig. 1E, high salt actually inhibited viral infection in cell lines with the long duration of treatment, which could be explained by our data showing that the treatment of cells with high salt for a long time can result in cell apoptosis, and therefore may inhibit viral replication.

2, Do type 1 interferon and viral infection regulate P97 acetylation?

Thanks for this good question! According to the reviewer's comment, we performed new experiments to observe the effect of IFN-I or viral infection on p97 acetylation. The results showed that neither IFN-I nor viral infection regulate p97 acetylation under the conditions of our experiments (please see new Fig. EV5L and EV5M**). We also attached the data here for your convenience:**

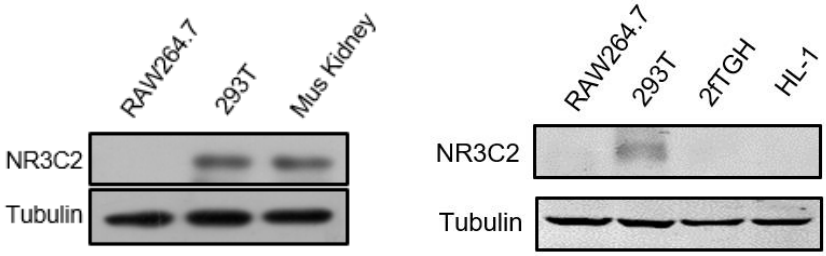


***Legend**: Immunoprecipitation analysis of pan-acetylation (Ace) of p97 in RAW264.7 cells treated with mIFNβ (500 IU/ml) (left) or VSV (MOI=1.0) (right) for indicated times.*

3, Aldosterone-mineralocorticoid receptor system serves as the major regulator of fluid homeostasis in vivo and is readily activated by the treatment of low salt diet in mice. The authors need to address whether aldosterone-mineralocorticoid receptor is important for the enhancement of anti-viral capacity by low-salt diet in mice.

Thanks! In this work, we clearly demonstrated by *in vitro* and *in vivo*

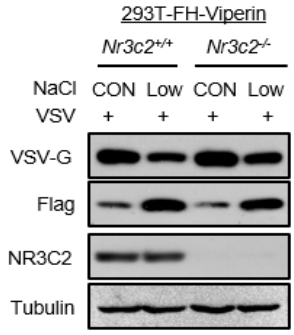
experiments that NaCl-mediated regulation of antiviral capacity is dependent on Viperin. In our studies in other projects, we demonstrated that Viperin protein cannot be produced in epithelial cells from almost all types of tissues (*Mol Cell*. 2020 Feb 20;77(4):734-747), whereas macrophages are the main type of cells to produced Viperin proteins during viral infection. We noticed that aldosterone-mineralocorticoid receptor majorly expresses in some epithelial tissues, including kidney, lung, blood vessel and so on. Consistently, in our experiments, we did not see the expression of the aldosterone-mineralocorticoid receptor NR3C2 in macrophages (please see the data below). Thus, given that Viperin and NR3C2 do not co-express in the same type of cells and that the extracellular osmolarity is not the major contributor to NaCl-mediated impairment of antiviral capacity, we speculate that aldosterone-mineralocorticoid receptor could not contribute a lot to NaCl-mediated regulation of antiviral capacity.



***Legend**: Western blot analysis of NR3C2 protein expression in RAW264.7, HEK293T, mouse primary kidney cells, 2fTGH and HL-1 cells.*

In addition, we further performed a new experiment to observe the role of the aldosterone-mineralocorticoid receptor NR3C2. Given that human embryonic kidney epithelial cell HEK293T can express NR3C2, but cannot produce Viperin proteins, we used a HEK293T stably expressing Flag-Viperin to observe the effect of NR3C2-knockout on low salt-mediated enhancement of antiviral capacity. The results showed that low salt treatment increased Flag-Viperin levels and restricted viral infection in *Nr3c2*$^{+/+}$ cells, whereas knockout of NR3C2 did not obviously change the effect of low salt on Viperin levels and viral infection, suggesting that the aldosterone-mineralocorticoid receptor NR3C2 is

dispensable for low salt-mediated enhancement of antiviral capacity. We thus added the new data and description in the new version (please see new Fig. EV5N and new Line 377-382**). We also attached it here for your convenience:**

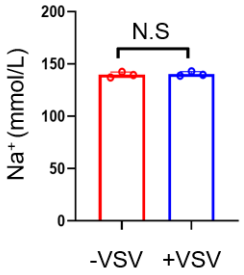


***Legend**: HEK293T cells with stable expression of FH-Viperin were used to make Nr3c2 $^{+/+}$ and Nr3c2 $^{-/-}$ cells using CRISPR-Cas9. Then cells were infected with VSV (MOI=1.0) in media containing normal (CON) or reduced (Low, -34 mM) concentration of NaCl for 12 hrs. VSV-G and FH-Viperin levels were analyzed by western blot.*

4, Is it possible that viral infection may change the local concentration of extracellular sodium and the extracellular osmolarity in the infected tissue?

We think that it is possible to change the extracellular sodium and osmolarity under certain conditions of viral infection. It has been reported that the volume-regulated anion channel (VRAC) controls cell volume by releasing Cl^-, other halide ions and organic osmolytes in response to cell swelling. A recent report demonstrated that viral infection-induced inflammatory cytokines can activate VRAC channels (Immunity. 2020 May 19;52(5):767-781**), suggesting that viral infection could change the release of Cl^- and organic molecules, thus changing the extracellular osmolarity.**

We feel that it will be very difficult to observe the accurate concentration of extracellular sodium and osmolarity in the infected tissues, since they are dynamically influenced by the interaction between viruses and the host, and different tissues and even the different sections of the same tissue have their specific responses. Despite this difficulty, we here observed the concentration of extracellular sodium in mouse blood after mice were infected with viruses for 48 hrs. The results showed no significant changes in the concentration of extracellular sodium under this condition (please see the data below**). However, we still believe there is a possibility that viruses could change (or momentarily change) the concentration of extracellular sodium and osmolarity at certain stages of infection.**

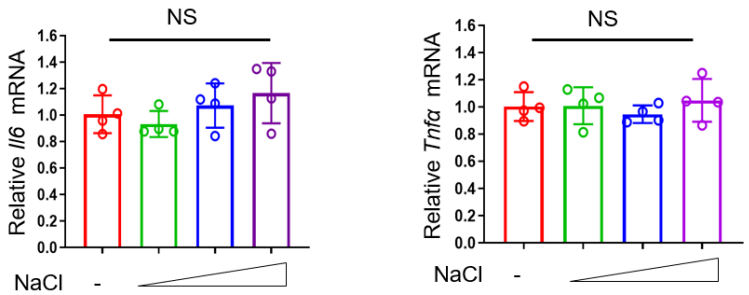


***Legend**: Mice were intraperitoneally infected with VSV (1x10^9 PFU per gram body, 48 hrs). Na$^+$ concentration in sera was analyzed by a Micro Blood Sodium Concentration Assay Kit.*

5, NaCl affects the universal ubiquitination in macrophages (Figure 5H-5J). What is the consequence? Does it affect any specific cellular physiology?

Thanks for this comment! In this study, we only focused on antiviral innate immunity of macrophages, since we aimed at exploring the effect of high salt on antiviral innate immune defense in this study. In addition to antiviral innate immunity, macrophages, which are recognized as the most plastic cells of the haematopoietic system with great functional diversity and are found in all tissues, have many other physiological functions, including development, homeostasis, tissue repair, immunomodulation, antigen presentation and so on (Nature. 2013 Apr 25;496(7446):445-55). Thus, it is really a huge task to explore the specific cellular physiological functions of macrophages that are affected by NaCl. We think that it will be interesting for researchers to study other effects of NaCl on macrophages under different physiological and pathological conditions in the future.

In spite of this difficulty, we performed a new experiment to observe whether NaCl affects the production of inflammatory cytokines in macrophages, which is an essential activity of macrophages to regulate many other functions. The results showed that treatment of cells with high salt for 12 hrs, which is a commonly used condition in our study and can inhibit universal ubiquitination in macrophages, did not significantly affect the production of the observed inflammatory cytokines, including IL-6 and TNF-α (please see the data below).



***Legend**: RT-qPCR analysis of Il6 or Tnfα mRNA levels in RAW264.7 cells treated with additional NaCl (+17, 34 and 51 mM) for 12 hrs.*

Based on these observations, we think that, those ubiquitinated proteins affected by NaCl could include both positive and negative regulators responsible for some signaling pathways and certain cellular physiological functions, which results in the balance or very minor changes in the overall cellular physiology. In addition, we speculate that NaCl-induced decrease in universal ubiquitination could be a continuous and gradual process. It could lead to the gradual decrease in normal cellular activity, which is able to be clearly observed only after treatment of cells with high salt for enough long time (such as more than 24 hrs).

We would like to point out that this is a good comment. Thus, we have added the corresponding descriptions to open this interesting question in the Discussion section of the new version (please see the new Line 480-484**). We also attached the description here for your convenience.**

"In addition, an interesting observation is that high salt affects the universal ubiquitination via p97 in macrophages. Thus, it will be attractive to study how the regulation of protein ubiquitination by high salt affects other physiological and pathological functions of macrophages in the future."

Thanks a lot for nice comments!

Dear Dr. Zheng,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, the referees now fully support the publication of your study.

Before we can proceed with formal acceptance, I have these editorial requests I ask you to address in a final revised manuscript:

- I would suggest this modified title:
High salt activates p97 to reduce host antiviral immunity by restricting Viperin induction

- Please restrict the key words on the title page to 5.

- There seems to be no callout for panel EV4O. Please check.

- 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 (main and EV figures), and that statistical testing has been done where applicable. Please avoid phrases like 'independent experiment', but clearly state if these were biological or technical replicates.

- Please remove the referee access information from the DAS and make sure that the PRIDE data is public upon publication of the paper.

- Please remove the sentence 'Expanded View for this article is available online' from the manuscript text file.

- As most Western blots shown are significantly cropped, please provide the source data for all the blots (main figures and EV figures). The source data will be published in a separate source data file per figure online along with the accepted manuscript and will be linked to the relevant figure. Please submit the source data (scans of entire blots) together with the revised manuscript. Please include size markers for scans of entire blots, label the scans with figure and panel number and send one PDF file per figure.

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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.

Kind regards,

Achim Breiling
Editor
EMBO Reports

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Authors have carefully and completely addressed all previous concerns. This manuscript will be of great interest to the readership of the EMBO Reports. I personally plan to do everything to decrease the amount of salt in my diet.

Referee #2:

The authors have satisfactorily responded to the points raised in my previous review.

Referee #3:

The authors have adequately addressed all my comments.

The authors have addressed all minor editorial requests.

Dr. Hui Zheng
Institutes of Biology and Medical Sciences
Soochow University
Ren-ai Road 199, Suzhou Industrial Park
Suzhou 215123
China

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1. Data

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.
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- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
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- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship 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.
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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
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- 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.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

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1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample size is described in respective figure legends.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	All animals were analyzed.
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For animal studies, include a statement about randomization even if no randomization was used.	The statement has been included, p23.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Animals were distributed into groups by animal caretakers unaware of the study design, p23.
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Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Statistically significant differences between groups were determined using two-tailed Student t-test when tha data exhibited normal distribution.
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C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Catalog numbers or clone numbers have been provided in the materials and methods section, p31-32.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK293T, A549, RAW264.7, HeLa, HT1080, 2fTGH, HepG2, HCT116 and Vero cells were obtained from the American Type Culture Collection, p25. All cell lines were regularly tested for mycoplasma contamination.

* for all hyperlinks, please see the table at the top right of the document

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E- Human Subjects

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15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The data for quantitative proteomic analysis generated in this study have been deposited to the ProteomeXchange Consortium via the PRIDE [1] partner repository with the dataset identifier PXD028645. The Raw data containing uncropped images of all gels and blots have been deposited in the Mendeley Data. The details can be found in the "Data Availability", p33.
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