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Expanding the host cell ubiquitylation machinery targeting cytosolic *Salmonella*

Mira Polajnar, Marina S. Dietz, Mike Heilemann, and Christian Behrends

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(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. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 30 January 2017

Thank you for the submission of your research manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, while the referees acknowledge that the findings are potentially interesting, they all point out that significant revisions are required and that the data need to be strengthened before the study can be considered for publication here. In particular, all referees request more experiments to further address the functional relationship between ARIH1, LRSAM1, and HOIP and its effect on the host's immune response and on bacterial replication.

From these comments it is clear that publication of the manuscript in our journal cannot be considered at this stage. On the other hand, given the potential interest of your findings, I would like to give you the opportunity to address the concerns and would be willing to consider a revised manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Basically, all points raised by the referees need to be addressed, and especially the evidence for the proposed SRIH1/LRSAM1/HOIP network needs to be strengthened.

I understand if you seek rapid publication elsewhere. Should you however decide to embark on such a revision, acceptance of the 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 or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

REFEREE REPORTS

Referee #1:

Ubiquitination is central for bacterial autophagy however underlying E3 ligases have not been fully catalogued nor characterized. The authors use an image-based RNAi screening approach to identify human RING-in-between-RING ubiquitin E3 ligase ARIH1 and its recognition of intracellular Salmonella for ubiquitination and bacterial autophagy.

The report is interesting with exciting potential but lacks novelty in its current format. The manuscript is suitable in length, can be more dense with results. This lab is highly suited to address all of my comments and advance this report beyond the discovery of another E3 ligase helpful in bacterial autophagy.

1. Introduction (p4): 'To date, only the E3 ligase LRSAM1 has been demonstrated to be involved in bacteria-associated ubiquitylation during infection with S. Typhimurium [12].' This is no longer true. Heath et al (Cell Reports, 2016), in which >600 E3 ligases were screened to discover RNF166, deserves mention and comparison to the results discovered here.

2. The authors conclude that ARIH1 function during bacterial infection is (i) independent of cullin RING ligases (CRLs) and (ii) shares redundancy with LRSAM1 (Figure 3). This is exciting biology that would benefit from in depth experimentation and validation. For example test for recruitment of ARIH1 / LRSAM1, perform survival assays.

3. The authors suggest that depletion of ARIH1 and LRSAM1 may trigger linear ubiquitination of Salmonella. Is this strictly due to HOIP? A more thorough investigation of HOIP vs ARIH1 / LRSAM1 can distinguish this work beyond previous reports on E3 ligases in bacterial autophagy.

4. It is proposed that linear ubiquitination of bacteria has other roles than xenophagic targeting. The authors test protein abundance of I B which is indicative of an activation of NF B signaling (Figure 3H). To substantiate this claim, quantify these difference across multiple blots. Other tests for NFkB signaling can also be easily performed.

Minor comments:
1. Figure 1A. Given the premise of this study, why does PARK2 not behave like ARIH1? This deserve some explanation in the text.

2. Figure 2D. Positive control?

3. p6: 'As expected, ARIH1 was unable to ubiquitylate S. Typhimurium that were stripped off their OMPs by proteinase K pretreatment (Figure 2D, first two lanes).' Replace with Figure 2E.

4. Figure 3A. Why do these control data significantly differ from controls in Figure 1E, F?

5. Figure 3D, F (and microscopy in general). Images will be more striking if use green / red / merge; it is difficult to distinguish green / blue clearly

Referee #2:

In this manuscript, Polajnar and Behrends identify ARIH1 as an E3 ligase that ubiquitylates cytosolic Salmonella within host cells. This is the main and sole clear conclusion of this work, nicely supported by the in vitro ubiquitylation assay. However, this is an important but not a major finding, as other E3 ligases (LRSAM1) have been shown to ubiquitylate cytosolic Salmonella. The authors also propose that ARIH1, LRSAM1 and HOIP form part of a network of E3 ligases that restrict proliferation of cytosolic bacteria and participates in the activation of host cell immune

responses. Regarding these more general and physiological conclusions, I find the data and explanations confusing and preliminary, and with several important shortcomings. It is also apparent that the required short format does not help to clearly describe all the experiments, its conclusions and limitations.

Major points:

1. In all infection experiments described in this work only one time-point (2 h after infection; or after addition of gentamicin?) is analyzed. The quantification of GFP-positive bacteria only at this time is insufficient to take conclusions about the effects of the different siRNA depletions on bacterial proliferation in the cytosol. How do we know that the siRNA treatments are not affecting invasion and therefore the intracellular levels of Salmonella? How do we know that the siRNA treatments are not affecting the stability of the SCV? This could affect the type of measurements done in this work. Therefore, these aspects must be controlled and the infection should be conveniently followed at different time-points to conclude about possible effects on proliferation of cytosolic bacteria.

2. I am not aware of the use of GFP expression under the control of the uhpT promoter as indicative of Salmonella that have escaped their SCVs. This is a critical point and there is no reference or experiment to firmly support and validate the use of this reporter.

3. The only experiment that is shown supporting a possible role of ARIH1, LRSAM1 and HOIP in the activation of host cell immune responses is Fig. 3H. At the very least, it is essential to quantify the apparently reduced levels of IkBalpha in cells depleted of ARIH and LRSAM1, but further experiments are needed to solidify this point.

4. It might be that indeed ARIH1, LRSAM1 and HOIP cooperate to ubiquitylate cytosolic Salmonella (the data presented suggests so), but as presented this is confusing. For example, judging from the different outcomes of depleting ARIH1 or LRSAM1 (Fig. 1F) or of depleting both ARIH1 and LRSAM1 (Fig. 3E), I would expect that the double ARIH1-LRSAM1 knock-down would have a much greater impact on M1-linked ubiquitin co-localizing with Salmonella than the single siRNA treatments. However, the effects shown in Fig. 3G are very similar between the different treatments. This aspect also needs to be better explained and/or solidified by additional experiments.

5. In the title, it is also implicit that ubiquitylation by ARIH1 targets Salmonella for autophagy. However, this is not formally shown in this work. In fact, the experiment in Fig. 3A even suggests that the role of ARIH1 is at least partially unrelated with autophagy.

Other points:

6. Considering the data presented, a more appropriate title would be "Expanding the host cell ubiquitylation machinery targeting cytosolic Salmonella". The only pathogen used here is Salmonella.

7. I would write simply "Salmonella" in the abstract and keywords and "S. enterica serovar Typhimurium (S. Typhimurium)" on line 4 of the introduction, and thereafter only S. Typhimurium or Salmonella.

8. Page 3, lines 4-8. This needs to be clarified. To my knowledge S. Typhimurium does not normally disseminate into the liver and spleen in humans (only in some mice strains, where it causes a systemic typhoid-like disease); on the other hand, S. Typhimurium normally causes a gastrointestinal disease in humans but not in mice.

9. Page 3, lines 9-14. This is incorrect. Please modify along the lines of "Invasion and intracellular proliferation are facilitated by various effector proteins delivered into host cells by two distinct type III secretion systems (T3SSs) encoded on the Salmonella pathogenicity islands 1 and 2 (SPI-1 and -2). In general, the SPI-1 T3SS enables invasion and stimulates the initial inflammatory response while the SPI-2 T3SS contributes to intracellular proliferation within the Salmonella-containing vacuole (SCV)".

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11. I could not find immunoblots (or other method) showing evidence of siRNA-mediated depletion of RNF144A or NKLAM on Fig. EV1, or did I miss something? Furthermore, siRNA-mediated depletion of at least IBRCD1 and RNF14 did not seem to work properly. Considering these siRNAs have been used in a primary screen, I do not find it essential that this is corrected experimentally, but it cannot be written "validated siRNAs" and these cases should be explained at least in the figure legends or in materials and methods.

12. There is apparently a different magnitude of effects between Fig. 1A (the primary screen) and Figs. 1E and 1F. Can this be explained by the way the data is presented? Furthermore, treatment with Parkin siRNA leads to about a 2-fold reduction in the numbers of GFP-positive Salmonella (Fig. 1A) when Parkin is apparently not expressed in HeLa cells (Fig. EV1 legend). This is bizarre.

13. Page 5, line 2, Sifs are introduced here without any need. The major phenotype associated with sifA mutant S. Typhimurium is indeed the progressive loss of the vacuolar membrane surrounding this bacterial strain.

14. However, I also do not understand the necessity or the rationale of using the sifA mutant (and not wild-type Salmonella). To my knowledge (see Fig. 5C of the cited BeuzÛn et al paper), at the time-point of infection (2 h) analysed there is no significant difference in the numbers of cytosolic bacteria between wild-type and sifA mutant S. Typhimurium. I am not claiming that all the experiments should be repeated with wild-type S. Typhimurium, but what is the evidence that at 2 h post-infection there are increased numbers of ubiquitylated cytosolic sifA mutant S. Typhimurium relative to wild-type bacteria? If analyzing further time-points, it would be better to use wild-type and not sifA mutant Salmonella.

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16. Page 6, line 1, should be Salmonella and not bacteria in the title of the section.

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22. Fig. EV4A. This is also shown in Fig. EV1B.

23. Please either describe its construction or indicate an appropriate reference for the S. Typhimurium strain used in this work.

24. In the legend of Fig. 1 and Fig. 2, I think it should be Fig. EV1 and Fig. EV2 (and not Fig. S1 and Fig. S2).

25. Fig. EV2 legend. Explain more clearly what s1, s2 and the positive control are.

26. Fig. 2. Indicate that the numbers represent kDa.

Referee #3:

In their manuscript, Polajnar and Behrends investigate the ubiquitylation of Salmonella Thyphimurium bacteria that have escaped in the cytosol taking advantage of a SifA mutant. They

performed a high-content analysis based on detection, in HeLa cells, of cytosolic bacteria that express GFP under the control of the uhpT promotor together with immunocytochemistry against ubiquitin ligases. Authors report that knock down of ARIH1 led to more cytosolic bacteria displaying less ubiquitin, in a comparable manner as what has been previously described for LRSAM1. Interestingly, authors have confirmed their data using a reconstituted in vitro system. They further investigated the effect of double KD for Atg7 and either ARIH1 or LRSAM1. They document that the double depletion led to a cell protection phenotype dependent and independent of autophagy. They report that ARIH1 ubiquitinates bacteria in a CRL-independent manner. On the other hand, they could not show additive effect of double ligase KD for ubiquitylation of cytosolic bacteria. Finally, they examined the effect of LRSAM1 KD on ARIH1 showing higher recruitment of the latter on bacteria, with similar observation upon HOIP KD.

The study is clearly presented and data are convincing. The finding of coordinated ubiquitylation involving several ligases is of interest in the field.

The only concern is that once the ligases identified, authors should better characterize the phenomenon they describe.

I would recommend first to document whether the recruitment of these ligases is similar or not, i.e. is there some polarity (in function of the escape from the membrane remnant for instance) or does the recruitment occur at multiple poles. The figure 4 suggests that some difference could be observed. Time point analysis could be performed (even better if movies are provided).

Second, it is not clear how the actions of the ligase are coordinated. One hint could be to performed imaging of the opposite experiment as those in Fig 4 (effect of ARIH1KD on HOIP and LRSAM1). Also in these experiments it would be interesting to follow in SIM (or better super-resolution methods, e.g. STORM, if available) the recruitment of ubiquitin upon KD of these ligases.

1st Revision - authors' response 29 April 2017

Please find enclosed the revised version of our manuscript (EMBOR-2016-43851V1) entitled "Expanding the host cell ubiquitylation machinery targeting cytosolic Salmonella Typhimurium," which we would like to re-submit as article to EMBO Reports. Thank you for your consideration of this manuscript. We are looking forward to hearing from you.

POINT-BY-POINT RESPONSE

Referee #1:

Ubiquitination is central for bacterial autophagy however underlying E3 ligases have not been fully catalogued nor characterized. The authors use an image-based RNAi screening approach to identify human RING-in-between-RING ubiquitin E3 ligase ARIH1 and its recognition of intracellular Salmonella for ubiquitination and bacterial autophagy. The report is interesting with exciting potential but lacks novelty in its current format. The manuscript is suitable in length, can be more dense with results. This lab is highly suited to address all of my comments and advance this report beyond the discovery of another E3 ligase helpful in bacterial autophagy.

1. Introduction (p4): 'To date, only the E3 ligase LRSAM1 has been demonstrated to be involved in bacteria-associated ubiquitylation during infection with S. Typhimurium [12].' This is no longer true. Heath et al (Cell Reports, 2016), in which >600 E3 ligases were screened to discover RNF166, deserves mention and comparison to the results discovered here.

A: We added a reference to this work in the text (p4). RNF166, however, was shown not to directly ubiquitylate *S.* Typhimurium but rather functions as a recruitment factor for autophagy receptors.

2. The authors conclude that ARIH1 function during bacterial infection is (i) independent of cullin RING ligases (CRLs) and (ii) shares redundancy with LRSAM1 (Figure 3). This is exciting biology

that would benefit from in depth experimentation and validation. For example test for recruitment of ARIH1 / LRSAM1, perform survival assays.

A: (i) We additionally tested the role of two CLRs – CUL1 and CUL3 - reported to interact and cooperate in the ubiquitylation of substrate [1]. Upon knockdown of either of these CLRs we observed no changes in the number of ubiquitylated cytosolic bacteria (new Fig 4B and EV2E-F). (ii) We performed colony formation unit (CFU) assays and automated quantification of GFP-positive *S*. Typhimurium upon single and combined knockdowns of ARIH1 and LRSAM1 at three different time points post infection (p.i.), namely 0.5, 2 and 6 hours (h). Intriguingly, the effect of the combined knockdown of ARIH1 and LRSAM1 led to a significant but smaller increase in GFP-positive bacteria than expected based on the phenotypes caused by individual depletion of either ligase (Fig 4G). Furthermore, when taking into account the whole intracellular population of S. Typhimurium, the double knockdown of ARIH1 and LRSAM1 did not further deteriorate the bacterial proliferation phenotype compared to single knockdowns (Figure 4H). These results suggest that ARIH1 and LRSAM1 have a positive, alleviating genetic interaction and likely act in the same anti-bacterial response pathway. Consistent with this notion, both ligases were found to increase linear ubiquitylation of cytosolic bacteria (rearranged Fig 5C and 5D), trigger activation of NFκB signaling (extended and new Fig 5 E-G) and have a patch-like colocalization on cytosolic bacteria (new Fig 2B and 2C). However, in contrast to LRSAM1, ARIH did not mediate recruitment of NDP52 (or of p62, LC3B and OPTN) (new Fig EV3B-I). Moreover, we observed striking differences in the recruitment kinetics of both E3 ligases to cytosolic bacteria: ARIH1 was recruited much earlier (0.5 h p.i.) than LRSAM1 while LRSAM1 persisted longer on S. Typhimurium (6 h p.i.) (new Fig 2D-E).

3. The authors suggest that depletion of ARIH1 and LRSAM1 may trigger linear ubiquitination of Salmonella. Is this strictly due to HOIP? A more thorough investigation of HOIP vs ARIH1 / LRSAM1 can distinguish this work beyond previous reports on E3 ligases in bacterial autophagy.

A: HOIP is the only ligase known to make linear chains. Thus, with the current knowledge we can assume that linear chains formation is a consequence of HOIP activity.

4. It is proposed that linear ubiquitination of bacteria has other roles than xenophagic targeting. The authors test protein abundance of IκBα which is indicative of an activation of NFκB signaling (Figure 3H). To substantiate this claim, quantify these difference across multiple blots. Other tests for NFkB signaling can also be easily performed.

A: An additional p.i. time point was added to this experiment (0.5 h p.i.) and an additional NFkB pathway marker (p-p65) was monitored (new Fig 5E). We also additionally performed quantification of the immunoblots for IκBα (new Fig 5F) and p-p65 (new Fig 5G).

Minor comments:

1. Figure 1A. Given the premise of this study, why does PARK2 not behave like ARIH1? This deserve some explanation in the text.

A: Parkin is not detectable in HeLa cells at the protein levels [2]. However, there is some transcription of its mRNA (based on the Human Protein Atlas). We added this in the figure legend (p27).

2. Figure 2D. Positive control?

A: We added the respective control (revised Fig 3D).

3. p6: 'As expected, ARIH1 was unable to ubiquitylate S. Typhimurium that were stripped off their OMPs by proteinase K pretreatment (Figure 2D, first two lanes).' Replace with Figure 2E.

A: We corrected this mistake.

4. Figure 3A. Why do these control data significantly differ from controls in Figure 1E, F?

A: Infection levels may vary depending on the confluency of the cells and the OD_{600} used for infection (OD_{600} used were between 1.7 and 2.4). Nevertheless, we repeated these assays and provided new figures with numbers comparable to those in Fig 1E and 1F (new Fig 4G).

5. Figure 3D, F (and microscopy in general). Images will be more striking if use green / red / merge; it is difficult to distinguish green / blue clearly

A: Images were replaced accordingly.

Referee #2:

In this manuscript, Polajnar and Behrends identify ARIH1 as an E3 ligase that ubiquitylates cytosolic Salmonella within host cells. This is the main and sole clear conclusion of this work, nicely supported by the in vitro ubiquitylation assay. However, this is an important but not a major finding, as other E3 ligases (LRSAM1) have been shown to ubiquitylate cytosolic Salmonella. The authors also propose that ARIH1, LRSAM1 and HOIP form part of a network of E3 ligases that restrict proliferation of cytosolic bacteria and participates in the activation of host cell immune responses. Regarding these more general and physiological conclusions, I find the data and explanations confusing and preliminary, and with several important shortcomings. It is also apparent that the required short format does not help to clearly describe all the experiments, its conclusions and limitations.

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A: We now performed GFP-positive bacterial counts and colony formation units (CFUs) assay at three different p.i. time points (i.e. time after the addition of gentamicin), namely 0.5, 2 and 6 h. We now show that at 0.5 h p.i. there are no significant differences between control and any of the knockdown samples (new Fig 4G and 4H), suggesting that infection and biogenesis of or escape from SCVs was not affected. Furthermore, we show that there is no difference in the number of GFP-positive bacteria at 2 h p.i. between sicontrol and mock treated samples again suggesting the preserved SCV integrity and infection levels (new Fig EV1B).

2. I am not aware of the use of GFP expression under the control of the uhpT promoter as indicative of Salmonella that have escaped their SCVs. This is a critical point and there is no reference or experiment to firmly support and validate the use of this reporter.

A: We apologize for this mistake. GFP expression is under control of a glucose-6-phosphat promoter. The wild-type strain (SFH2) was reported in [3]. The corresponding D*sifA* strain (SFH4) was constructed accordingly in the lab of Dirk Bumann. This information was added in the Methods section.

3. The only experiment that is shown supporting a possible role of ARIH1, LRSAM1 and HOIP in the activation of host cell immune responses is Fig. 3H. At the very least, it is essential to quantify the apparently reduced levels of IkBalpha in cells depleted of ARIH and LRSAM1, but further experiments are needed to solidify this point.

A: An additional p.i. time point was added to this experiment (0.5 h p.i.) and an additional NFkB pathway marker (p-p65) was monitored (new Fig 5E). We also additionally performed quantification of the immunoblots for IκBα (new Fig 5F) and p-p65 (new Fig 5G).

4. It might be that indeed ARIH1, LRSAM1 and HOIP cooperate to ubiquitylate cytosolic Salmonella (the data presented suggests so), but as presented this is confusing. For example, judging from the different outcomes of depleting ARIH1 or LRSAM1 (Fig. 1F) or of depleting both ARIH1 and LRSAM1 (Fig. 3E), I would expect that the double ARIH1-LRSAM1 knock-down would have a much greater impact on M1-linked ubiquitin co-localizing with Salmonella than the single siRNA treatments. However, the effects shown in Fig. 3G are very similar between the different treatments. This aspect also needs to be better explained and/or solidified by additional experiments.

A: The automated quantification was repeated several times and the difference between single and double knockdowns were consistently similar. The finding that the effect of the combined ligase knockdown led to a significant but smaller increase than expected based on the phenotypes caused by individual depletion of either ligase (with regard to numbers of GFP-positive bacteria, CFU and linear Ub (new Fig 4G-H and reordered Fig 5C-D) indicates that both ARIH1 and LRSAM1 share a positive, alleviating genetic interaction and thus, are likely to function in the same anti-bacterial pathway.

5. In the title, it is also implicit that ubiquitylation by ARIH1 targets Salmonella for autophagy. However, this is not formally shown in this work. In fact, the experiment in Fig. 3A even suggests that the role of ARIH1 is at least partially unrelated with autophagy.

A: We tested the recruitment of four different autophagy markers (LC3B, p62, NDP52, and OPTN) that were all predominately unchanged upon knockdown of ARIH1 (new Fig EV3B-I). The connection of ARIH1 and LRSAM1 in xenophagy is now discussed in the text in more detail. We also modified the title of our manuscript.

Other points:

6. Considering the data presented, a more appropriate title would be "Expanding the host cell ubiquitylation machinery targeting cytosolic Salmonella". The only pathogen used here is Salmonella.

A: We adjusted the title as suggested.

7. I would write simply "Salmonella" in the abstract and keywords and "S. enterica serovar Typhimurium (S. Typhimurium)" on line 4 of the introduction, and thereafter only S. Typhimurium or Salmonella.

A: This was corrected.

8. Page 3, lines 4-8. This needs to be clarified. To my knowledge S. Typhimurium does not normally disseminate into the liver and spleen in humans (only in some mice strains, where it causes a systemic typhoid-like disease); on the other hand, S. Typhimurium normally causes a gastrointestinal disease in humans but not in mice.

A: The sentence was corrected and added that this was shown only in mice.

9. Page 3, lines 9-14. This is incorrect. Please modify along the lines of "Invasion and intracellular proliferation are facilitated by various effector proteins delivered into host cells by two distinct type III secretion systems (T3SSs) encoded on the Salmonella pathogenicity islands 1 and 2 (SPI-1 and -2). In general, the SPI-1 T3SS enables invasion and stimulates the initial inflammatory response while the SPI-2 T3SS contributes to intracellular proliferation within the Salmonella-containing vacuole (SCV)".

A: This paragraph was corrected.

10. Page 17, should be "involved".

A: We corrected this mistake.

11. I could not find immunoblots (or other method) showing evidence of siRNA-mediated depletion of RNF144A or NKLAM on Fig. EV1, or did I miss something? Furthermore, siRNA-mediated

depletion of at least IBRCD1 and RNF14 did not seem to work properly. Considering these siRNAs have been used in a primary screen, I do not find it essential that this is corrected experimentally, but it cannot be written "validated siRNAs" and these cases should be explained at least in the figure legends or in materials and methods.

A: We fully agree with this. anti-RNF144A and -NKLAM antibodies did not work in our hands. We modified the sentence accordingly and wrote an additional explanation in the figure legends.

12. There is apparently a different magnitude of effects between Fig. 1A (the primary screen) and Figs. 1E and 1F. Can this be explained by the way the data is presented? Furthermore, treatment with Parkin siRNA leads to about a 2-fold reduction in the numbers of GFP-positive Salmonella (Fig. 1A) when Parkin is apparently not expressed in HeLa cells (Fig. EV1 legend). This is bizarre.

A: The magnitude of effects was indeed higher in the screen, which could be contributed to the fact that pooled siRNAs were used. Moreover, the calculated Z-score does not show a fold change but rather calculates how many standard deviations a score differs from the mean. In the case of Parkin any changes were statistically insignificant.

13. Page 5, line 2, Sifs are introduced here without any need. The major phenotype associated with sifA mutant S. Typhimurium is indeed the progressive loss of the vacuolar membrane surrounding this bacterial strain.

A: The sentence was corrected.

14. However, I also do not understand the necessity or the rationale of using the sifA mutant (and not wild-type Salmonella). To my knowledge (see Fig. 5C of the cited Beuzón et al paper), at the time-point of infection (2 h) analyzed there is no significant difference in the numbers of cytosolic bacteria between wild-type and sifA mutant S. Typhimurium. I am not claiming that all the experiments should be repeated with wild-type S. Typhimurium, but what is the evidence that at 2 h post-infection there are increased numbers of ubiquitylated cytosolic sifA mutant S. Typhimurium relative to wild-type bacteria? If analyzing further time-points, it would be better to use wild-type and not sifA mutant Salmonella.

A: In parallel to our screen with the Δ*sifA* strain, we performed an additional screen with wild-type *S*. Typhimurium. However, none of the 14 RBR ligases in the wild-type screen scored as hit (i.e. Z-scores for the number of GFP-positive and the percentage of Ub- and GFP-positive bacteria >+2 and <-2, respectively) (new Fig EV1C-E).

15. In Fig. 1A, the absolute average numbers of GFP+ Salmonella and of Ub+/GFP+ Salmonella for siRNA control should be indicated in the figure legend.
A: The numbers were provided in the figure legends as suggested.

16. Page 6, line 1, should be Salmonella and not bacteria in the title of the section.

A: This was corrected.

17. Which strain of S. Typhimurium was used for the in vitro ubiquitylation assay?

A: The same strain was used throughout all experiments: cytoGFP Δ*sifA*. We tried to make this clearer in the text.

18. Not essential, but in Fig. 2D it would be nice to also have the "no wash" condition.

A: We added this.

19. By the inspection of Fig. EV2C alone it does not look like MLN4924 blocks all CRL activity in cells. This should be more clearly explained.

A: The experiment was repeated and the Western blot was replaced to show that the neddylation of CUL1 is blocked when MLN4924 is added (new Fig EV2C).

20. Simply based on its mentioning in the text and legend, I cannot understand Fig. EV3B.

A: We tried to explain this more clearly in the figure legend.

21. First line of the discussion: it should be "ligase"

A: This was corrected.

22. Fig. EV4A. This is also shown in Fig. EV1B.
A: The difference is that in Fig EV1A the siRNAs used were pooled while in Fig EV5A it was only a single siRNA targeting HOIP. We tried to explain this more clearly in the figure legends.

23. Please either describe its construction or indicate an appropriate reference for the S. Typhimurium strain used in this work.

A: We apologize for this default. The wild-type strain (SFH2) was reported in [3] and the expression of GFP is under control of a glucose-6-phosphate promoter. The corresponding D*sifA* strain (SFH4) was constructed accordingly in the lab of Dirk Bumann. We add this information to the Material and Method section.

24. In the legend of Fig. 1 and Fig. 2, I think it should be Fig. EV1 and Fig. EV2 (and not Fig. S1 and Fig. S2).

A: This was corrected.

25. Fig. EV2 legend. Explain more clearly what s1, s2 and the positive control are.

A: We tried to explain it more clearly in the text and the figure legend.

26. Fig. 2. Indicate that the numbers represent kDa.

A: This was corrected.

Referee #3:

In their manuscript, Polajnar and Behrends investigate the ubiquitylation of Salmonella Thyphimurium bacteria that have escaped in the cytosol taking advantage of a SifA mutant. They performed a high-content analysis based on detection, in HeLa cells, of cytosolic bacteria that express GFP under the control of the uhpT promotor together with immunocytochemistry against ubiquitin ligases. Authors report that knock down of ARIH1 led to more cytosolic bacteria displaying less ubiquitin, in a comparable manner as what has been previously described for LRSAM1. Interestingly, authors have confirmed their data using a reconstituted in vitro system. They further investigated the effect of double KD for Atg7 and either ARIH1 or LRSAM1. They document that the double depletion led to a cell protection phenotype dependent and independent of autophagy. They report that ARIH1 ubiquitinates bacteria in a CRL-independent manner. On the other hand, they could not show additive effect of double ligase KD for ubiquitylation of cytosolic bacteria. Finally, they examined the effect of LRSAM1 KD on ARIH1 showing higher recruitment of the latter on bacteria, with similar observation upon HOIP KD.

The study is clearly presented and data are convincing. The finding of coordinated ubiquitylation involving several ligases is of interest in the field.

The only concern is that once the ligases identified, authors should better characterize the phenomenon they describe.

I would recommend first to document whether the recruitment of these ligases is similar or not, i.e. is there some polarity (in function of the escape from the membrane remnant for instance) or does the recruitment occur at multiple poles. The figure 4 suggests that some difference could be observed. Time point analysis could be performed (even better if movies are provided).

Second, it is not clear how the actions of the ligase are coordinated. One hint could be to performed imaging of the opposite experiment as those in Fig 4 (effect of ARIH1KD on HOIP and LRSAM1). Also in these experiments it would be interesting to follow in SIM (or better super-resolution methods, e.g. STORM, if available) the recruitment of ubiquitin upon KD of these ligases.

A: In line with these suggestions, additional imaging was performed using conventional and super-resolution microscopy (dSTORM). A patch-like recruitment for both endogenous ARIH1 and LRSAM1 could be observed by both microscopy techniques at 2 h p.i. (new Fig 2B,2C, 2D and Fig EV6D). Furthermore, by following the recruitment kinetics of endogenous ARIH1 and LRSAM1 at three different p.i. time points, we were able to show that ARIH1 was recruited already at 0.5 h p.i. and persisted on bacteria until 2 h p.i. (new Fig 2D and 2E). At this latter time point, LRSAM1 was recruited to the cytosolic *S.* Typhimurium and both of the E3 ligases colocalized on the surface of the same bacterium. At a later time point (6 p.i.) both of the ligases were largely depleted from the surface of *S.* Typhimurium, although the localization of LRSAM1 to the bacteria was more pronounced compared to ARIH1.

Furthermore, dSTORM imaging was used to image the recruitment of ARIH1 upon knockdown of LRSAM1 and oppositely, the recruitment of LRSAM1 upon ARIH1 knockdown. A similar nanoscale patch-like recruitment for both ligases was observed as in control cells (new Fig EV6F and EV6G). However, in contrast to the increased recruitment of ARIH1 in LRSAM1 depleted cells (reordered Fig 6A and 6B), no significant differences were detected in the colocalization of LRSAM1 in ARIH1 depleted cells (new Fig D-E). These new results are discussed in the text. Notably, since endogenous antibodies were used for imaging throughout our experiments, the recruitment of HOIP on cytosolic bacteria could not be observed due to the lack of a suitable antibody that could immunodetect endogenous HOIP.

References

1. Scott DC, Rhee DY, Duda DM, Kelsall IR, Olszewski JL, Paulo JA, de Jong A, Ovaa H, Alpi AF, Harper JW*, et al.* (2016) Two Distinct Types of E3 Ligases Work in Unison to Regulate Substrate Ubiquitylation. *Cell* **166:** 1198-1214 e1124

2. Nagaraj N, Wisniewski JR, Geiger T, Cox J, Kircher M, Kelso J, Paabo S, Mann M (2011) Deep proteome and transcriptome mapping of a human cancer cell line. *Mol Syst Biol* **7:** 548

3. Spinnenhirn V, Farhan H, Basler M, Aichem A, Canaan A, Groettrup M (2014) The ubiquitin-like modifier FAT10 decorates autophagy-targeted Salmonella and contributes to Salmonella resistance in mice. *J Cell Sci* **127:** 4883-4893

2nd Editorial Decision 26 May 2017

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, all referees are positive about the study and request only minor changes to clarify text and figures. Moreover, referee 1 points out two recent papers that should be cited and discussed as appropriate. Referee 2 recognizes the use of the sifA mutant as a convenient model but suggests to verify the basic observation that ARIH1 ubiquitinates cytosolic Salmonella with a wildtype strain. Upon further discussion with the other referees they agree on this point. Referee 1 suggested to monitor the recruitment of ARIH1 to wildtype bacteria, similar to the experiment shown in Fig. 2A to address this point.

Finally, please include a more detailed description of the sifA mutant strain either in the methods or supplement or by adding a reference, as outlined by referee 2. Please also make sure to specify the use of this strain in the respective figure legends as appropriate (e.g. Fig 3C) to rule out any ambiguity.

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study.

- Statistics: you note in the figure legends that you quantified "XXX cells/sample". Please also indicate the number of samples that were analyzed (n=).

- We noticed some inconsistencies between the figure legends and the figure that need your attention, e.g., Fig. 6f is not mentioned in the legend, while panel "h" does not exist in the figure. Likewise, Fig. EV3 lists "j" which is not present in that figure.

We look forward to seeing a final version of your manuscript as soon as possible.

REFEREE REPORTS

Referee #1:

The manuscript has been significantly revised and is a clear advance. The coordination of specific ubiquitin chains and novel / multiple E3 ligases to cytosolic bacteria is very timely, and of great interest to the field. This report would strongly benefit from a commentary to highlight its specific contribution among other recent, exciting reports (eg Heath et al, Cell Reports, 2016; Noad et al, Nat Microbiol, 2017; van Wijk et al, Nat Microbiol, 2017).

Minor comments:

1. (p3) 'Anti-bacterial autophagy (xenophagy) serves as an important adaptive immunity mechanism against invasive intracellular bacteria [6].'
The term '....important adaptive immunity mechanism...' is misleading here. '...serves as a cell-autonomous immune mechanism against invasive intracellular bacteria' can be more suitable.

2. The Introduction (p4) should be updated with 2 recent papers (Noad et al, Nat Microbiol, 2017; van Wijk et al, Nat Microbiol, 2017) also describing ubiquitination of Salmonella. The interpretation of NFKB results (eg p11 bottom of first paragraph) and Disucssion can also be updated given these new reports.

3. Fig 1B, C. Inserting a horizontal dotted line at Z=2 and also Z=-2 across the graph would help readers to appreciate significant results.

4. Fig 2B, C and Fig 6F, G. Super resolution microscopy, as requested by reviewers 3, is a nice addition to the manuscript. However the merging of super res images on top of blurry bacterial images (not super resolution) does not help here. Instead of using a 'merge' image, please provide an enlargement of ARIH1 / LRSAM1 (similar to how Heilemann as previously done in van Wijk et al, Nat Microbiol, 2017) to clearly illustrate patches.

5. Fig 3C. Add a wash ' + + + + ' row just above the gel to be consistent with Fig 3D and 3E.

6. Fig 5D Y axis. Should be 'M1+ / GFP+-Salmonella'.

Referee #2:

I do appreciate the amount of high quality work the authors put in this revised manuscript (and how they compacted it within the 25,000 characters, which I thought it was not possible). While most of my concerns have been addressed, I still have a number of issues with the manuscript:

- I understand why the sifA mutant was used in the initial screen, but I do not understand why subsequent experiments were not done with wild-type bacteria. Even if there are less wild-type bacteria in the cytosol of host cells than sifA mutant bacteria, those fewer cytosolic wild-type bacteria should also be significantly less ubiquitinated after ARIH1 knock-down. Of course, the authors are looking at how the host ubiquitination machinery recognises bacterial pathogens and the sifA mutant is just a convenient model. However, the basic observation (ARIH1 is involved in the ubiquitination of cytosolic Salmonella) should be experimentally confirmed with wild-type bacteria. Alternatively, at the very least, it should be highlighted in the discussion that the findings were

based on sifA mutant Salmonella as a model and an explanation should be given for why no effect (Figs EV1C-E) was observed with the wild-type strain.

- The source of the sifA mutant should be explicitly indicated (i.e., obtained from Dirk Bumann). Please confirm that it is indeed a transposon mutant. I would have expected it was obtained by lambda red-mediated deletion. Ideally, ask Dirk for a precise description of how it was constructed and characterized (can be complemented by sifA in trans?) and add this to the paper (e.g. to the supplemental material), or provide a reference where this has been done. I did not search Dirk's papers extensively but could not find a reference to the strain. I noticed this strain has been used as model in other papers related with bacterial cytosolic recognition/ubiquitination with only the vague mention of "sifA mutant obtained from Dirk Bumann", which in my view is not enough for a model bacterium. Should be nice to have this sorted.

- Mentions to "bacterial replication" or even "bacterial proliferation" should be avoided. Best to use only increase/decrease in bacterial numbers. Definitely, please do replace the labelling of Figs. 4C-F by "fold relative to 30 min p.i." (or something similar) as "fold replication" in that context is an error.

- I think Salmonella alone would be enough in the title.

- Some italics are missing in Salmonella or in the S. of S. Typhimurium.

Referee #3:

Authors gave satisfactory answers to the points raised by this reviewer. Hence, as far as this reviewer is concerned, the manuscript can be processed further towards publication.

2nd Revision - authors' response 06 June 2017

RESPONSE TO EDITOR

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below. As you will see, all referees are positive about the study and request only minor changes to clarify text and figures. Moreover, referee 1 points out two recent papers that should be cited and discussed as appropriate. Referee 2 recognizes the use of the sifA mutant as a convenient model but suggests to verify the basic observation that ARIH1 ubiquitinates cytosolic Salmonella with a wildtype strain. Upon further discussion with the other referees they agree on this point. Referee 1 suggested to monitor the recruitment of ARIH1 to wildtype bacteria, similar to the experiment shown in Fig. 2A to address this point.

>We performed the requested experiment and observed that ARIH1 is also recruited to wild-type bacteria (please also see response to referee 2).

Finally, please include a more detailed description of the sifA mutant strain either in the methods or supplement or by adding a reference, as outlined by referee 2.

>We provided this information in the method section (please also see response to referee 2).

Please also make sure to specify the use of this strain in the respective figure legends as appropriate (e.g. Fig 3C) to rule out any ambiguity.

>We checked all figure legends. Fig 3 was the only one in which the strain identity was missing. We now added this information.

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study.

- Statistics: you note in the figure legends that you quantified "XXX cells/sample". Please also indicate the number of samples that were analyzed (n=).

>We provided the missing information.

- We noticed some inconsistencies between the figure legends and the figure that need your attention, e.g., Fig. 6f is not mentioned in the legend, while panel "h" does not exist in the figure. Likewise, Fig. EV3 lists "j" which is not present in that figure.

We corrected this mix-up.

POINT-BY-POINT RESPONSE

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The manuscript has been significantly revised and is a clear advance. The coordination of specific ubiquitin chains and novel / multiple E3 ligases to cytosolic bacteria is very timely, and of great interest to the field. This report would strongly benefit from a commentary to highlight its specific contribution among other recent, exciting reports (eg Heath et al, Cell Reports, 2016; Noad et al, Nat Microbiol, 2017; van Wijk et al, Nat Microbiol, 2017).

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1. (p3) 'Anti-bacterial autophagy (xenophagy) serves as an important adaptive immunity mechanism against invasive intracellular bacteria [6].' The term '....important adaptive immunity mechanism...' is misleading here. '...serves as a cell-autonomous immune mechanism against invasive intracellular bacteria' can be more suitable.

We thank the reviewer for this suggestion. This has been changed.

2. The **Introduction** (p4) should be updated with 2 recent papers (Noad et al, Nat Microbiol, 2017; van Wijk et al, Nat Microbiol, 2017) also describing ubiquitination of Salmonella. The interpretation of NFKB results (eg p11 bottom of first paragraph) and **Disussion** can also be updated given these new reports.

The two papers are now mentioned in the introduction and the discussion.

3. Fig 1B, C. Inserting a horizontal dotted line at Z=2 and also Z=-2 across the graph would help readers to appreciate significant results.

We added the lines as suggested.

4. Fig 2B, C and Fig 6F, G. Super resolution microscopy, as requested by reviewers 3, is a nice addition to the manuscript. However the merging of super res images on top of blurry bacterial images (not super resolution) does not help here. Instead of using a 'merge' image, please provide an enlargement of ARIH1 / LRSAM1 (similar to how Heilemann as previously done in van Wijk et al, Nat Microbiol, 2017) to clearly illustrate patches.

The figures were changed accordingly.

5. Fig 3C. Add a wash ' + + + + ' row just above the gel to be consistent with Fig 3D and 3E.

Done.

6. Fig 5D Y axis. Should be 'M1+ / GFP+-Salmonella'.

We corrected this.

Referee #2:

I do appreciate the amount of high quality work the authors put in this revised manuscript (and how they compacted it within the 25,000 characters, which I thought it was not possible). While most of my concerns have been addressed, I still have a number of issues with the manuscript:

- I understand why the sifA mutant was used in the initial screen, but I do not understand why subsequent experiments were not done with wild-type bacteria. Even if there are less wild-type bacteria in the cytosol of host cells than sifA mutant bacteria, those fewer cytosolic wild-type bacteria should also be significantly less ubiquitinated after ARIH1 knock-down. Of course, the authors are looking at how the host ubiquitination machinery recognises bacterial pathogens and the sifA mutant is just a convenient model. However, the basic observation (ARIH1 is involved in the ubiquitination of cytosolic Salmonella) should be experimentally confirmed with wild-type bacteria. Alternatively, at the very least, it should be highlighted in the discussion that the findings were based on sifA mutant Salmonella as a model and an explanation should be given for why no effect (Figs EV1C-E) was observed with the wild-type strain.

We performed the requested consensus experiment and monitored recruitment of ARIH1 to cytosolic wild-type bacteria. Indeed, we found that ARIH1 colocalizes with wild-type bacteria in the cytosol. We added this new result as Fig EV2B.

- The source of the sifA mutant should be explicitly indicated (i.e., obtained from Dirk Bumann). Please confirm that it is indeed a transposon mutant. I would have expected it was obtained by lambda red-mediated deletion. Ideally, ask Dirk for a precise description of how it was constructed and characterized (can be complemented by sifA in trans?) and add this to the paper (e.g. to the supplemental material), or provide a reference where this has been done. I did not search Dirk's papers extensively but could not find a reference to the strain. I noticed this strain has been used as model in other papers related with bacterial cytosolic recognition/ubiquitination with only the vague mention of "sifA mutant obtained from Dirk Bumann", which in my view is not enough for a model bacterium. Should be nice to have this sorted.

After consulting with Dirk Bumann from who we obtained the sifA strain it turned out that this mutant was indeed generated by lambda red-mediated depletion. We deeply apologize for not having checked this earlier. We now provided the requested information in the Materials & Method section.

- Mentions to "bacterial replication" or even "bacterial proliferation" should be avoided. Best to use only increase/decrease in bacterial numbers. Definitely, please do replace the labelling of Figs. 4C-F by "fold relative to 30 min p.i." (or something similar) as "fold replication" in that context is an error.

We edited the text and figures as requested.

- I think Salmonella alone would be enough in the title.

We changed the title accordingly.

- Some italics are missing in Salmonella or in the S. of S. Typhimurium.

We corrected these cases.

Referee #3:

Authors gave satisfactory answers to the points raised by this reviewer.
Hence, as this reviewer is concerned, the manuscript can be processed further towards publication.

We thank the reviewer for her/his positive feedback!

3rd Editorial Decision 14 June 2017

Thank you for the submission of your revised manuscript to EMBO reports and for incorporating all changes requested by the referees and myself. I am now writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once the following correction has been made:

Thank you for adding the number of analysed samples in the figure legends. Browsing through the figure legends again, I noticed that in many cases the number of biological replicates was only two (Fig. 1E, F; 2E, H, I; 4B, C-H; 6E; EV2A; EV3B-I). We note that statistical measures applied to too small a sample size are not significant and can suggest actually a false level of significance. In particular, if n <3 the use of statistical tests is not appropriate and I would therefore ask you to remove the p-values from those graphs and only display the SD.

If all remaining corrections have been attended to, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

3rd Revision - authors' response 18 June 2017

The authors uploaded a revised version of their manuscript file.

4th Editorial Decision 22 June 2017

I have gone through the modified figure legends and I fear that there was a misunderstanding. I apologize for not being more explicit.

You have done most of the experiments with 3-4 technical replicates and with 2-3 biological replicates, which is perfectly fine. The figures now show a representative experiment with the standard deviation and the p-values calculated over the technical replicates. You have removed the p-values in those cases, where the number of biological replicates was <2. The data is now clearly labeled, however, it is statistically not correct to calculate the p-value over technical replicates. Replicates, while being an important internal control for the performance of your experiments, cannot be used for statistics and p-values. The statistics should come from independent biological samples (see also http://embor.embopress.org/content/13/4/291).

Given that most of your experiments were performed with three independent biological replicates, could you please re-calcuate the statistics using these biological replicates? In cases where n<3 (and n refers to biological replicates) no error bars or p-values should be displayed but the actual data points be shown with the median or mean value. Could you please change the figures and statistics accordingly? Thank you very much.

4th Revision - authors' response 26 June 2017

The authors made the requested changes and uploaded the final version of their manuscript.

5th Editorial Decision 30 June 2017

Thank you for updating the statistics and graphs. I am very pleased to accept your manuscript for publication in EMBO reports. Thank you for your contribution to our journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Christian Behrends
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2016-43851V1

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

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.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs 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 and any statistical test employed should be justified
- 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.

2. Captions

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.
- definitions of statistical methods and measures:
  - 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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com	Antibodypedia
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http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm	MRC Guidelines on animal use
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http://biomodels.net/miriam/	MIRIAM Guidelines
http://jjj.biochem.sun.ac.za	JWS Online
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was dependent on what was being measured or detected (screening, WB quantification, colocalization events) based on previously published studies.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	There was no exlusion of samples from analyses.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Normal distribution of data was assumed based on the sample size (n>30) and that sample mean and variance are independent.
Is there an estimate of variation within each group of data?	Yes, ANOVA test was used.
Is the variance similar between the groups that are being statistically compared?	Yes, ANOVA test was used.

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).	The antibodies used in this study are listed in the Method section on p.14.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The cell line was not recently autenticated but is mycoplasma negative.

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA

14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under '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	There is no additional deposition of data.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No.