EMBO reports

Manuscript EMBO-2016-43841

Shigella hijacks the glomulin-cIAPs-inflammasome axis to promote inflammation

Shiho Suzuki,Toshihiko Suzuki, Hitomi Mimuro, Tsunehiro Mizushima, Chihiro Sasakawa

Corresponding author: Shiho Suzuki, Tokyo Medical and Dental University & Chihiro Sasakawa, The University of Tokyo

Review timeline:

Submission date:	19 December 2016
Editorial Decision:	25 January 2017
Revision received:	25 September 2017
Editorial Decision:	16 October 2017
Revision received:	25 October 2017
Accepted:	06 November 2017

Editor: Achim Breiling

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

1st Editorial Decision 25 January 2017

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, referees #2 and #3 acknowledge the potential interest of the findings, whereas referee #1 is more critical. All three referees have raised a number of concerns and suggestions to improve the manuscript, or to strengthen the data and the conclusions drawn, which need to be addressed during a revision. In particular, we think that points 2 (further mutations tot the RING domain - see also the comments of referee #3), 3 (provide data with alternative loss of function methods, or provide further data that the KD or the transfections where efficient) and 8 (construction of GLMN-null macrophages by Crispr/Cas9) of referee #1 and all points by the other two referees need to be addressed with further data.

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 a complete 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 or rejection 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; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient

for the revisions so that we can discuss the revisions further.

Supplementary/additional data: 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 labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Important: All materials and methods should be included in the main manuscript file.

We now 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. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

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.

REFEREE REPORTS

Referee #1:

This manuscript is a follow up to a 2014 PNAS paper by the same authors proposing Shigella, via injection of a bacterial E3 ligase, promotes inflammasome activation in macrophages and cell death. While the concepts put forth by the authors are intriguing, unfortunately the manuscript does not appear to be a sufficient advance to merit publication in EMBO Reports.

Major comments:

1. The rationale for the study is not straighforward. The authors searched for IpaH7.8 interacting partners that also directly bind to GLMN: such an experimental strategy is inherently biased and may miss the real targets, for example related to other GLMN or IpaH7.8 functions.

2. From the GLMN side, the interactions with cIAP RING domains are difficult to rationalize. GLMN, like other inhibitors of RING domains (e.g. Salmonella SOPA binding to TRIM56 and 65) bind very specifically to select RING domains, based on very specific structural contacts. In fact, as mentioned by the authors, the literature has shown that such inhibitory proteins do not bind to other RING domains. Although the authors attempt to address this by sequence analysis, I do not understand the logic. For reasons that are not explained, the authors focus on 4 of the several residues that differ between the Glmn binding RING from Rbx1 and the non-binding RING from Rbx2 and select a single serine as the basis for specificity, yet this serine is not in the GLMN binding site. cIAP1 and cIAP2 RING domains are structurally different from that from GLMN's sole known target Rbx1, and yet there is no explanation for how the structures of GLMN and cIAP-family RING domains could interact specifically. Confidence is further diminished by choice of mutants used in Figure 4D.

3. From the cIAP side, it is also difficult to undertand how there could be interactions because IAP E3 ligases are autoinhibited, with their RING domains blocked by other domains. This is consistent with the minimal activity in the in vitro cIAP2 E3 ligase assays (e.g. Fig. 3E and G). Auto inhibition might also explain why there is minimal E3 ligase activity in the in vitro assays.

4. Throughout the entire manuscript the authors employ siRNA approaches with no regard for efficiency of transfection or experiment-to-experiment variation. Macrophages (even CSF-1 generated BMDMs) have a plethora of nucleic acid surveillance mechanisms that could confound the data.

5. The methods (lines 378-384 state that SiO2 etc. were used to stimulate the NLRP3 inflammasome. However, this reviewer is under the impression that such an experimental approach is impossible without prior stimulation of the IL-1b mRNA via TLR agonists (it is unclear how the authors did this with the Shigella infections). How do the authors explain their data if the substrate for the inflammasome does not exist?

6. For the Shigella infections of macrophages, the authors pre-treat the cells with E. coli LPS. Why? How is this relevant to Shigella infection of intestinal macrophages? What changes to iAP/GLMN could LPS pre-treatment enforce?

7. In 1D, the authors conclude that cIAPs 'co-localize' with GLMN (and perhaps ASC and the rest of the inflammasome). However, such a conclusion cannot be readily drawn, as the formation of the ASC 'speckle' is a giant supra-molecular aggregate that probably coalesces many proteins.

8. Without data from a complete GLMN-deficient macrophage population (that could generated by Crispr/Cas9 or potentially through in vitro generation of macrophages from fetal levers of GLMN-/- embryos or by Crispr/Cas9) most of the conclusions drawn are dependent on siRNA and inferred outcomes. Direct in-cell evidence is essential to conclude GLMN has a key role in inflammasome formation (even in the system studied here).

Referee #2:

This is an interesting manuscript that identifies a novel molecular mechanism by which Shigella promotes inflammasome activation. The authors identify glomulin as an inhibitor of the cIAP-inflammasome axis and show that the Shigella effector IpaH7.8 ubiquitinates glomulin and targets it for proteosomal degradation to promote cIAP-dependent efficient inflammasome activation.

The data is of high quality and the results are stringent. I have a few comments that need to be addressed to strengthen the quality of this manuscript.

1. Does mutating the key Serine residue in the RING domain of cIAP1/2 impair their catalytic activity, specifically their auto-Ub and Ub of caspase-1?

2. In the discussion, please address what bacterial effectors in Salmonella and Pseudomonas might control the GLMN-cIAP-casp1 axis.

3. In Fig.3G, was a high stringency buffer used for the IP? otherwise, the Ub signal could be that of cIAP2 or any of its co-immunoprecipitated associated partners. If a high stringency buffer was used, this needs to be indicated in the methods section under IP.

4. In the abstract, line 41, please replace the sentence "suggests that the GLMN-cIAPs axis...negative regulator" to "suggests that GLMN is a negative regulator of cIAP-mediated inflammasome activation"

5. In the intro, lines 61-62, please add a mention of "gasdermin D"as the key caspase-1 substrate leading to pyroptosis.

6. In the discussion, line 251, please correct the typo "pyroptisis" to "pyroptosis"

7. In the discussion, line 257-258, please add the relevant references e.g. for gut homeostasis (Dagenais et al. Mucosal Immunology 2016), for Immunity (Bertrand et al. Immunity 2009).

Referee #3:

This is a succinct, well written manuscript describing the interaction of IpaH7.8, an effector protein from Shigella, with the host protein glomulin (GLMN). The authors show that IpaH7.8 which is a known E3 ubiquitin ligase ubiquitinates GLMN, and this has effects on activation of the inflammasome during infection of macrophages via the interact of GLMN with cIAP1 and cIAP2. This is a novel pathway for modulating the inflammasome in cells.

The figures are well presented and of high quality, and the data justify the conclusions of the authors by and large., Some controls are missing from the report which should be included in any revised manuscript.

Fig 1C/3G: control pull down with GFP alone missing.
Fig 2AB was XIAP knock down effective in these cells?
Line 138: give genotype of S325 here
For the serine to alanine version of cIAP1 and 2, were these proteins expressed at levels comnparable to the wild-type proteins. Did the authors construct other substitutions (ie C or D instead of A), as the alanine substitution appeared to lead to a loss of cIAP function and results that were counter to their model for the effect of GLMN on cIAP.

The authors should be careful about using the word affinity at several points in their manuscript. The did not measure any affinities of interactions in their work, merely the capacity of over-expressed proteins to pull down others inside cells.

1st Revision - authors' response 25 September 2017

Referee #1 (Report for Author)

This manuscript is a follow up to a 2014 PNAS paper by the same authors proposing Shigella, via injection of a bacterial E3 ligase, promotes inflammasome activation in macrophages and cell death. While the concepts put forth by the authors are intriguing, unfortunately the manuscript does not appear to be a sufficient advance to merit publication in EMBO Reports.

Major comments:

1. The rationale for the study is not straighforward. The authors searched for IpaH7.8 interacting partners that also directly bind to GLMN: such an experimental strategy is inherently biased and may miss the real targets, for example related to other GLMN or IpaH7.8 functions.

We do not agree that our approach was biased and would potentially miss the “real targets”. Our experimental approach was to identify host binding partners of bacterial IpaH E3 ligase effectors. Our approach has been extensively adopted as a well-established method for studying bacterial strategies to circumvent host innate immune responses (Ashida et al., Curr Opin Microbiology, 2017; Suzuki et al., PNAS, 2014; Ashida et al., Nat Rev Microbiol, 2014; PLoS Pathog, 2013; Kobayashi et al., Cell Host Microbe 2013; Sanada et al., Nature 2012). Of course, we cannot rule out the possibility of there being GLMN or IpaH7.8 binding partners other than cIAPs, since protein–protein interactions during bacterial infection of host cells are complex and highly dynamic. However, in this study, we uncovered the functional link between GLMN and inflammasome activation during *Shigella* infection of macrophages, a critical step for bacteria to egress from macrophages. Therefore, we believe that our experimental approach involving GST-pulldown screening followed by the Y2H system is an appropriate strategy.

2. *From the GLMN side, the interactions with cIAP RING domains are difficult to rationalize. GLMN, like other inhibitors of RING domains (e.g. Salmonella SOPA binding to TRIM56 and 65) bind very specifically to select RING domains, based on very specific structural contacts. In fact, as mentioned by the authors, the literature has shown that such inhibitory proteins do not bind to other RING domains. Although the authors attempt to address this by sequence analysis, I do not understand the logic. For reasons that are not explained, the authors focus on 4 of the several residues that differ between the Glmn binding RING from Rbx1 and the non-binding RING from*

Rbx2 and select a single serine as the basis for specificity, yet this serine is not in the GLMN binding site. cIAP1 and cIAP2 RING domains are structurally different from that from GLMN's sole known target Rbx1, and yet there is no explanation for how the structures of GLMN and cIAP-family RING domains could interact specifically. Confidence is further diminished by choice of mutants used in Figure 4D.

Reviewer #1 claims that the serine of interest is not located at the binding site of GLMN and RING, but this has previously been reported by Duda and colleagues (Mol Cell 2012). Duda and colleagues performed structural analysis of the interaction between RING and GLMN using a monomeric model and showed that inhibition of binding of the RING E3 ligase occurs by masking of the E2-binding surface. Importantly, as will be mentioned in our following response, cIAPs have been suggested to form and function as dimers. If the dimeric form was active *in vivo*, it is reasonable to speculate that some additional, yet uncharacterized, interface may be involved in the interaction between GLMN and cIAPs. Furthermore, we cannot rule out the possibility that GLMN and RING ligases might also interact and function as multimers, or undergo post-translational modification, which might also contribute to the interactions between them. Therefore, we feel that answering the reviewer's comment is outside the scope of this type of study.

3-1. *From the cIAP side, it is also difficult to understand how there could be interactions because IAP E3 ligases are autoinhibited, with their RING domains blocked by other domains. This is consistent with the minimal activity in the in vitro cIAP2 E3 ligase assays (e.g. Fig. 3E and G). Auto inhibition might also explain why there is minimal E3 ligase activity in the in vitro assays.*

As suggested, cIAP1 and cIAP2 exhibit two conformations, namely a monomer auto-inhibited form and a RING domain-mediated dimerization form (Feltham et al., 2011 JBC; Fig.7 on Budhidarmo & Day, 2015, Seminars in Cell & Developmental Biology). Previous studies showed that the monomeric form of cIAPs was unstable and resulted in the RING domains being blocked by other domains. Importantly, cIAP dimerization is essential for the E3 ligase activity of cIAP1 and cIAP2, because monomeric RING mutants (deficient in dimer formation) are incapable of interacting with the ubiquitin-charged E2 enzyme, which is required for autoubiquitylation. The above literature indicated that the RING domains are exposed only with dimeric cIAPs, suggesting that GLMN specifically interacts with dimeric cIAPs. In any case, we believe that elucidation of the molecular details of the interaction between GLMN and cIAPs, together with inflammasomes, is an interesting and challenging route of investigation, but is not a focus of the current study.

3-2. *Throughout the entire manuscript the authors employ siRNA approaches with no regard for efficiency of transfection or experiment-to-experiment variation.*

We investigated the knockdown efficiency of the target genes, as we consider this more meaningful than the transfection efficiency, and presented the results in the expanded view of Figure 2 A and B. Concerning experiment-to-experiment variation, we checked the knockdown efficiencies in triplicate to confirm reproducibility (see the Methods section: Statistical analyses).

3-3. *Macrophages (even CSF-1 generated BMDMs) have a plethora of nucleic acid surveillance mechanisms that could confound the data.*

We agree and therefore in all of the experiments using siRNA-knockdown cells, we included a non-targeting siRNA control along with the gene targeting siRNA.

3-4. *The methods (lines 378-384 state that SiO2 etc. were used to stimulate the NLRP3 inflammasome. However, this reviewer is under the impression that such an experimental approach is impossible without prior stimulation of the IL-1b mRNA via TLR agonists (it is unclear how the authors did this with the Shigella infections). How do the authors explain their data if the substrate for the inflammasome does not exist?*

Thank you for raising this concern and we apologize that this was unclear. Macrophages were pre-treated with LPS to upregulate IL-1b mRNA prior to stimulation of inflammasome. In the revised manuscript we have corrected this (see lines 409–410). Similarly, prior to *Shigella* infection, macrophages were pre-treated with LPS to upregulate IL-1b mRNA as shown in lines 396–397.

4-1. *For the Shigella infections of macrophages, the authors pre-treat the cells with E. coli LPS. Why? How is this relevant to Shigella infection of intestinal macrophages?*

The aim of pre-treatment of cells with LPS was to upregulate IL-1b mRNA via stimulation of TLR *in vitro*. This is a well-established method for the *in vitro* assessment of inflammasome activity in macrophage*s*.

4-2. *What changes to iAP/GLMN could LPS pre-treatment enforce?*

Upon LPS treatment, the GLMN signal is relocalized into punctate structures as shown in Fig 5 of Suzuki et al., 2014. In addition, we observed the localization of cIAPs with or without LPS treatment by immunohistochemical analysis and found that LPS pre-treatment did not affect the localization of cIAPs. To make this clear in the manuscript, we added the immunohistochemical imaging data in the Expanded View Figure (see Fig EV1, bottom panel).

5. *In 1D, the authors conclude that cIAPs 'co-localize' with GLMN (and perhaps ASC and the rest of the inflammasome). However, such a conclusion cannot be readily drawn, as the formation of the ASC 'speckle' is a giant supra-molecular aggregate that probably coalesces many proteins.*

From our work and that of others, the ASC pyroptosome is not thought to be a protein aggregate. We observed the co-localization of the ASC pyropyosome together with several other proteins related to the inflammasome pathway or the outcome of the cell death pathway in macrophages, but not all of them co-localized (even the inflammasome component proteins such as the NLRs and the active form of caspase-1). This observation provides evidence against the possibility that the ASC speckle is a giant aggregate of multiple proteins.

Our investigations to establish the role of GLMN relied heavily on the validity of the GLMN antibody, which we created in our previous study (see supplemental information for Suzuki et al, 2014 PNAS). To confirm this, we performed immunoblotting and immunohistochemical analysis of GLMN in $GLMN^{+/+}$ and $GLMN^{+/-}$ macrophages. The strong signals in $GLMN^{+/+}$ cells became weaker in $GLMN^{+/-}$ macrophages, validating the specificity of the GLMN antibody.

6. *Without data from a complete GLMN-deficient macrophage population (that could generated by Crispr/Cas9 or potentially through in vitro generation of macrophages from fetal levers of GLMN-/- embryos or by Crispr/Cas9) most of the conclusions drawn are dependent on siRNA and inferred outcomes. Direct in-cell evidence is essential to conclude GLMN has a key role in inflammasome formation (even in the system studied here).*

Concerning the generation of GLMN-null cells from fetal livers, we previously attempted such experiments and the GLMN-deficient embryos died at embryonic day E7.5–E10.5 (Suzuki et al., PNAS 2014), suggesting that the GLMN-deficient embryos did not undergo development of the liver. Indeed, an earlier study had reported that knocking out the *GLMN* gene in mice embryos was lethal (Arai et al. PNAS 2003). Therefore, it is no surprise that we failed to obtain fetal livers in GLMN-KO mice.

To address the referee's comment and to reinforce our findings and those of previous studies, we tried to generate GLMN-KO, cIAP1-KO, cIAP2-KO, cIAP1 and 2 double-KO, and XIAP-KO cells. Using an efficient method of CRISPR/Cas9 genome editing, we were able to obtain cIAP1-KO, cIAP2-KO, cIAP1 and 2 double-KO, and XIAP-KO cells, but not GLMN-KO cells. In the case of the cIAP-KOs, the cells obtained were a heterologous population consisting of homozygous null cells and heterozygous cells of the target genes, and a few WT cells. By contrast, in the case of the GLMN-KO, the cells obtained were a population consisting of heterozygous cells and WT cells, suggesting that GLMN-deficient cells result in a lethal phenotype.

Furthermore, we performed siRNA-mediated GLMN knockdown in GLMN heterozygous cells as an alternative method to see if we could generate GLMN-null cells. Under the conditions of high knockdown efficiency, transfectants aggregated, and appeared floating in the medium (these macrophages had lost their adhesiveness), and were not undergoing active growth. These observations provided further evidence that GLMN deficiency is either fatal or unable to support cell growth. One possible cause of the GLMN-deficient phenotype may be related to cell cycle arrest, similar to the CUL1 correlation with cell cycle progression. With a comparatively low

knockdown efficiency, we may be able to obtain viable cells, but this would only be possible under conditions of cells expressing GLMN at a low level.

Referee #2 (Report for Author)

This is an interesting manuscript that identifies a novel molecular mechanism by which Shigella promotes inflammasome activation. The authors identify glomulin as an inhibitor of the cIAP-inflammasome axis and show that the Shigella effector IpaH7.8 ubiquitinates glomulin and targets it for proteosomal degradation to promote cIAP-dependent efficient inflammasome activation.

The data is of high quality and the results are stringent. I have a few comments that need to be addressed to strengthen the quality of this manuscript.

We appreciate the referee's comments to improve our manuscript. We have modified our manuscript according to the referee's suggestions.

1. Does mutating the key Serine residue in the RING domain of cIAP1/2 impair their catalytic activity, specifically their auto-Ub and Ub of caspase-1?

This is an interesting question and it would be valuable to understand the ubiquitination of caspase-1 or other inflammasome-related proteins such as NLRs and ASC. Although we tried to detect the ubiquitination of these proteins using several commercially available antibodies, we were unable to detect the ubiquitination of caspase-1. At present, however, we cannot rule out the possibility that the antibodies used would be eligible to detect the K63 ubiquitination of caspase-1.

2. In the discussion, please address what bacterial effectors in Salmonella and Pseudomonas might control the GLMN-cIAP-casp1 axis.

We have addressed this issue in the modified manuscript. In the case of *Salmonella,* SspH2 effector is homologous to *Shigella* IpaH7.8, and both effectors share a similar molecular structure, suggesting that they likely share similar molecular interactions and functions. In the case of *Pseudomonas*, some *Pseudomonas* spp. produce putative IpaH7.8-like proteins. However, to our knowledge*, P. aeruginosa* does not possess an effector protein homologous to IpaH7.8, although screening for such an effector may be worthwhile.

We have addressed this point by adding the following sentences to the discussion section in the modified manuscript: "In our study, other pathogenic bacteria such as *Salmonella* and *Pseudomonas* also showed cIAP-mediated inflammasome activation on infection of macrophages (Fig EV2C). Some pathogenic bacteria are also known to possess bacterial effectors that are similar to *Shigella* IpaHs. For example, the *Salmonella* effector SspH2, is homologous to *Shigella* IpaH7.8 and shares a similar molecular structure with IpaH7.8. SspH2 may also function in controlling the GLMN–cIAPs–casp1 axis, and this is a subject for future analysis."

3. In Fig.3G, was a high stringency buffer used for the IP? otherwise, the Ub signal could be that of cIAP2 or any of its co-immunoprecipitated associated partners. If a high stringency buffer was used, this needs to be indicated in the methods section under IP.

The buffer used for cell lysis and washing the beads for IP in Fig 3G is described in the Materials & Methods section on lines 370–372 and 382. We cannot confirm the stringency of the buffer (Sigma CelLytic M buffer), since its composition is not specified by the manufacturer.

Regarding the signal for cIAP2-ubiquitination in the bottom image (immunoblotting of IP-myc) in Fig 3G, we speculate that it is not due to the binding of co-immunoprecipitated proteins. In the IP assay, the precipitated beads were mixed with SDS-PAGE sample buffer (10% glycerol, 0.1% Bromophenol Blue, 62.5 mM Tris-HCl, pH 6.8, 5% 2-mercaptoethanol, and 2% SDS), and then boiled at 100°C for 6 min. Following this treatment, the immunoprecipitated proteins would have lost their three-dimensional structures and also their ability for protein–protein interactions, since the sample buffer contained strong detergent and disulfide reducing agent. If some co-immunoprecipitated proteins existed in the sample, they would not affect the results of immunoblotting.

We apologize for omitting this information in the original manuscript. We have added this information to the Materials and Methods section of the modified manuscript to avoid any confusion.

4. In the abstract, line 41, please replace the sentence "suggests that the GLMN-cIAPs axis...negative regulator" to "suggests that GLMN is a negative regulator of cIAP-mediated inflammasome activation"

We have replaced the sentence on line 41.

5. In the intro, lines 61-62, please add a mention of "gasdermin D"as the key caspase-1 substrate leading to pyroptosis.

Thank you for raising this point. We have added some statements in the revised text mentioning *"gasdermin D"* (Kayagaki *et al.*, 2015, Nature, Shi *et al*., 2015, Nature). See lines 63–70.

6. In the discussion, line 251, please correct the typo "pyroptisis" to "pyroptosis"

Apologies for this error, the spelling has been corrected.

7. In the discussion, line 257-258, please add the relevant references e.g. for gut homeostasis (Dagenais et al. Mucosal Immunology 2016), for Immunity (Bertrand et al. Immunity 2009).

Thank you, we have cited the relevant publications in the revised manuscript.

Referee #3 (Report for Author)

This is a succinct, well written manuscript describing the interaction of IpaH7.8, an effector protein from Shigella, with the host protein glomulin (GLMN). The authors show that IpaH7.8 which is a known E3 ubiquitin ligase ubiquitinates GLMN, and this has effects on activation of the inflammasome during infection of macrophages via the interact of GLMN with cIAP1 and cIAP2. This is a novel pathway for modulating the inflammasome in cells.

The figures are well presented and of high quality, and the data justify the conclusions of the authors by and large., Some controls are missing from the report which should be included in any revised manuscript.

We appreciate the referee's comments to improve our manuscript. We have modified our manuscript according to the referee's suggestions.

Fig 1C/3G: control pull down with GFP alone missing.

In Fig 1C, the leftmost lane shows the control pull down with GFP beads alone, which is the co-precipitate with anti-GFP-conjugated beads without expression of 6myc-cIAPs or GFP-IpaH7.8CA. The control pull down with GFP-IpaH7.8CA expression only is shown in the second lane from the left. This lane shows the co-precipitate with anti-GFP-conjugated beads with expression of GFP-IpaH7.8CA.

In Fig 3G, we performed immunoprecipitation with anti-myc antibody and protein G beads. Note that it is not a pull down assay using GFP-beads (please refer to the legend of Fig 3G). The immunoprecipitation was performed to confirm the cIAP ubiquitination signal by condensing the myc-tagged cIAPs. We apologize for any confusion regarding this. To clarify, we have rewritten the relevant text in the Materials and Methods section.

Fig 2AB was XIAP knock down effective in these cells?

Yes. XIAP was effectively knocked down, as shown in Fig 2A and B. We confirmed the knockdown efficiency by RT-PCR as shown in Fig EV2A.

Line 138: give genotype of S325 here

In the revised manuscript, we added "(*mxiA*::Tn*5*)" as the genotype of S325. See line 152 in the revised version.

For the serine to alanine version of cIAP1 and 2, were these proteins expressed at levels comnparable to the wild-type proteins. Did the authors construct other substitutions (ie C or D instead of A), as the alanine substitution appeared to lead to a loss of cIAP function and results that were counter to their model for the effect of GLMN on cIAP.

We constructed a serine-to-glutamic acid (E) version of cIAP1 and 2, as well as the alanine (A) substitution. Using clones including these newly-obtained point mutants (RING domains of cIAPs in pGEX-6P expression vector, or the 6myc-tagged full-length cIAPs in mammalian expression vectors), we repeated the experiments in Fig 4 (GST pull down assay) and Fig 5 (over-expression study of cIAPs).
In the GST pull down assay, as shown in Fig 4E, the cIAP RING domains, in which the serine residue of interest was replaced with a glutamic acid, did not interact with GLMN. We similarly investigated the interaction of GLMN with serine-to-alanine point mutants.
In the cIAP over-expression study, the serine-to-glutamic acid mutant of cIAP failed to activate inflammasomes, as seen with the serine-to-alanine point mutants (Fig 5A–C). We added these data to Fig 4E and Fig 5A–C.

The authors should be careful about using the word affinity at several points in their manuscript. The did not measure any affinities of interactions in their work, merely the capacity of over-expressed proteins to pull down others inside cells.

Thank you for this comment. In the revised manuscript, we have replaced the word "affinity" in lines 238, 245, and 249.

2nd Editorial Decision 16 October 2017

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the referees that were asked to re-evaluate your study (you will find enclosed below). As you will see, the referees now support the publication of your manuscript in EMBO reports. Referee #1 suggests a final modification of the discussion, which we ask you to do in a final revised version of the manuscript.

Further, I have the following editorial requests that also need to be addressed:

Please provide the three EV as .xls or .doc files. Then, please remove their legends from main MS file and include the legends in the respective EV table file, thus either as text in the .doc file, or as tab in the .xls file.

We now strongly encourage the publication of original source data (in particular of Western blots) 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. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

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

REFEREE REPORTS

Referee #1:

The authors answered most of the key questions. The authors should consider being more speculative in their discussion of the binding mechanism because of differences between IAP and RBX RING domains. But I don't think they can do much more at this point without more detailed analysis of the GLMN binding mechanism or an inducible knockout system. Because of the functional importance of the targeting, the manuscript is suitable for publication.

Referee #2:

The authors have addressed my concerns and the paper is now suitable for publication.

Referee #3:

The authors have addressed my concerns. I am happy for the article to be published.

2nd Revision - authors' response 25 October 2017

Referee #1:

The authors answered most of the key questions. The authors should consider being more speculative in their discussion of the binding mechanism because of differences between IAP and RBX RING domains. But I don't think they can do much more at this point without more detailed analysis of the GLMN binding mechanism or an inducible knockout system. Because of the functional importance of the targeting, the manuscript is suitable for publication.

Thank you for your comment to improve our manuscript. We have modified our manuscript (lines 230, 240, 258) according to the referee's suggestion.

Referee #2:

The authors have addressed my concerns and the paper is now suitable for publication.

We thank referee for the comments.

Referee #3:

The authors have addressed my concerns. I am happy for the article to be published.

We thank referee for the comments.

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: Shiho Suzuki, Chihiro Sasakawa
Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2016-43841V1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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 $\chi 2$ 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.

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?	All experimental methods performed in this study were pre-established from previously published studies or pilot experiments and planned accordingly.
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. Statistical analysis used are described in Materials and Methods section on p.20.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Such distributions were not tested.
Is there an estimate of variation within each group of data?	See Materials and Methods section, Statistical analysis on p.20.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	Yes. See Materials and Methods section, Statistical analysis on p.20.

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 described in the Method section on p.14-15.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All of the cell lines used in this study were not recently authenticated. Cells are 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 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	NA. There is no 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. 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

22. 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.