EMBO reports

# Deubiquitinating enzymes UBP12 and UBP13 stabilize the brassinosteroid receptor BRI1

Yongming Luo, Junpei Takagi, Lucas Claus, Chao Zhang, Shigetaka Yasuda, Yoko Hasegawa, Junji Yamaguchi, Libo Shan, Eugenia Russinova, and Takeo Sato
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## Transaction Report:

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

Dear Dr. Sato

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

As you will see, the referees acknowledge that the findings are potentially interesting and overall well supported by the data. However, they also point out several concerns and have a number of suggestions for how the study should be strengthened, which should be addressed.

Given these supportive and constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. 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.

We invite you to submit your manuscript within three months of a request for revision. This would be October 1st in your case. However, we are aware of the fact that many laboratories are not fully functional due to COVID-19 related shutdowns and we have therefore extended the revision time for all research manuscripts under our scooping protection to allow for the extra time required to address essential experimental issues. Please contact us to discuss the time needed and the revisions further.

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1) A data availability section is missing.
2) Your manuscript contains error bars based on n=2. Please use scatter blots showing the individual datapoints in these cases. The use of statistical tests needs to be justified.

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1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

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6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Please note that a Data Availability section at the end of Materials and Methods is now mandatory. In case you have no data that requires deposition in a public database, please state so instead of refereeing to the database.
See also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

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The following points must be specified in each figure legend:
- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
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10) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

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Yours sincerely

Martina Rembold, PhD
Senior Editor
EMBO reports

**********

Referee #1:

In this manuscript by Luo et al., the authors aim to show the role of two deubiquitinating enzymes, UBP12 and UBP13, in brassinosteroid signaling. First, they generate an inducible RNAi transgenic line in the ubp13 mutant background targeting UBP12. They perform physiological measurements of this line on regular medium and in the presence of brassinolide. They then use western blotting to track the levels of the BRI1 protein in this line in the presence of brassinolide or an inhibitor of vacuolar trafficking. They next perform protein interaction studies between UBP12 and BRI1 and ubiquitin and BRI1.Then they cross their

inducible line into multiple lines expressing wild-type BRI1 or BRI1 with modifications to its ubiquitin status. Finally, they examine the subcellular localization of BRI1 in the RNAi inducible line.

From these experiments the authors conclude that UBP12 and UBP13 participate in brassinosteroid signaling through the prevention of ubiquitin mediated endocytosis and degradation of BRI1. They also conclude that UBP12 and UBP13 are able to cleave K63-linked polyubiquitin chains on BRI1 that has been previously shown to be mediated by the E3 ubiquitin ligase, PUB13 (also confirmed here). The complementary approaches and detailed analyses provide solid evidence for their conclusions. There a just a few minor concerns regarding the manuscript that are detailed below:

1. It was previously shown that the same UBP proteins interact with BZR1 (Wang et al. Molecular and Cellular Proteomics 2013). This work appears to be absent from mention in the manuscript but may indicate an additional role for UBPs in controlling BR signaling. This should at least be mentioned in the discussion including how this would relate to the work presented.

2. The inducible RNAi transgenic line seems like a useful tool since the double knockout of ubp12 and ubp13 is not viable. That being said, there are additional genetic resources (stable RNAi, partial loss-of-function alleles) that may be useful to complement the studies here. One might worry the inducible system could introduce variable expression depending on the experimental conditions. This would be difficult to account for. It would be nice to see additional expression data showing that the RNAi is equally effective at suppressing UBP12 expression in all of the genetic backgrounds presented. Also, the brssinolide treatment experiments could be performed with other alleles to confirm the results presented.

3. The dwarf phenotypes of the ubp12i/ubp13 plants in the light are striking, similar to the bri1 mutant, and are used to indicate that the BR pathway is compromised. This does not seem to be the case in the dark (Figure S3). While the light grown plants resemble the bri1 mutant, the dark grown plants seem less severe than expected for a bri1 mutant grown in the dark. Unfortunately, the bri1 mutant was not included as a control in the dark grown, brassinolide treatment, experiment. The bri1 control and its responsiveness should have been included for reference in this experiment.

Referee #2:

In their manuscript the authors address the role of two members of the Arabidopsis UBP deubiquitinase family in the regulation of BRI1 homeostasis.
BRI1 sorting and proteolytic turnover appears to be mediated by reversible poly-ubiquitination, involving activity of PUB E3 ubiquitin ligases. Here, the authors provide evidence for an antagonistic function for UBP12 and UBP13 mediating BRI1 deubiquitination and stabilization.

Several points support the authors' claim:

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) ubp12i/ubp13 shows reduced protein abundance of BRI1, which can be reverted by concanamycin A - a potent inhibitor of V-type ATPases and vacuolar cargo sorting.

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) Perhaps the most compelling evidence for UBP12/13 functioning in BRI1 deubiquitination is provided by expression of ubiquitination-deficient bri1-25KR in ubp12i/ubp13, causing reversion of ubp12i/ubp13 growth defects. This observation is accentuated further by quantification of mCitrine-tagged BRI1 and bri1-25KR reporter signals, revealing increased vacuolar targeting, specifically of wild type BRI1, when expressed in ubp12i/ubp13.

Overall, the data presented make a very strong case for a critical role of deubiquitinase activity in the modulation of plasma membrane protein activity in Arabidopsis. These findings add substantially to our understanding of ubiquitination-related signaling in higher plants.

There is one thing the authors need to consider:

In Figure 3, the authors present their analysis of deubiquitination activity of UBP13 in vitro. I was intrigued by Fig. 3D, suggesting PUB13 enzymatic activity in formation of K63-linked polyubiquitin chains. Whilst still not entirely resolved, some elements participating in K63-linked polyubiquitin chain formation appear fairly conserved in eukaryotes. RING-finger type E3 ligases seem to be involved as well as a unique type of E2 heterodimers composed of Ubc13 (UBC35/36 in Arabidopsis) and UEVs. Apart from that, additional E3 ligases have been implicated in K63-linked polyubiquitin chain formation, such as certain HECT proteins. There is also circumstantial evidence, suggesting that PUB13 might catalyze formation of such polyubiquitin chains, given its role in sorting control of plasma membrane protein. However, to my knowledge, no clear biochemical proof has been provided so far.
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Related to that: By employing FK2 (an IgM, recognizing polyubiquitin chains, preferentially when attached to substrate proteins) and Apu3 (which preferentially recognizes K63-linked polyubiquitin chains), the authors came to the conclusion that K63-linked chains represent in vitro substrates for UBP13. I would recommend testing for K48-linked chains as well (e.g. by employing Apu2), to learn about the configuration of the poly-ubiquitin chains produced in their in vitro ubiquitination assays (Fig. 3).

Referee #3:

The work by Luo et al provides clear evidence for the involvement of two deubiquitinating enzymes, UBP12 and UBP13, in regulating brassinosteroid (BR) signaling via mutant analysis. The manuscript may be further improved by showing the effects of UBP12 and/or UBP13 overexpression on BRI1 ubiquitination and stability and BR response. Some outstanding questions include: how does brassinolide (BL) affect UBP12/13 association with BRI1? How does brassinolide affect UBP12/13 mediated deubiquitination of BRI1?

Further points to consider:
Figure 1B - How does hypocotyl length of the ubp mutants compare to bri1 mutants +/- BL? What about the effect of BL on hypocotyl length of light grown mutant seedlings compared to WT? The amount of BL used in some assays (500/1000) seems high.

Figure 1 - The level of dephosphorylated BES1 in ubp12i/ubp13 mutant #1-7 (fig 1D) is not significantly different from WT in the presence of BL. However, the response of #1-7 to BL is significantly different from that of wild type (fig 1B). While ubp12i/ubp13 mutant #12-8 has significantly less dephosphorylated BES1 but the BL response is not as drastic as shown for #1-7. How is this explained?

Figure 1C - identify phosphorylated and unphosphorylated BES1 on anti-BES1 blot.

Figure 2A - bands requires quantification, compare BRI1 levels in mutant to WT.

Figure 2 - Is it possible to look at the abundance/stability of BRI1 in the absence of protein synthesis?

Figure 3D and E - Demonstrate deubiquitination in vivo by overexpressing UBP12/13 or at least 'semi' in vivo - isolate BRI1 from planst and use as substrate for deubiquitination.

Figure 4 - what is the response of the transgenic plants to BL compared to WT? BRI125KR-mCit/bri1/ubp12i/ubp13 seems to be smaller and have less lateral roots compared to BRI125KR-mCit/bri1.Is it possible to quantify size/root growth.

Figure 5A - The difference in fluorescence intensity between WT and ubp12i/ubp13 is not clear. Compare to WT, ubp12i/ubp13 is expected to show less plasma membrane localized BRI1 due to increase internalization and degradation, this is not apparent in the figures. Further assays are needed to better demonstrate increased vacuolar targeting.

Some minor edits:
Line 50-51, requires editing for clarity.

Line 52-54, requires references.

Line 55, "The antagonistic process of ubiquitination" should read (?) "The antagonistic process to ubiquitination" or "The antagonistic process of deubiquitination"

Line 63, Ewan et al, 2011 demonstrated deubiquitinating activity for UBP12 (not UBP13).

Line 124, "the most active BR, complements the dwarf phenotype of ubp12i/ubp13." I don't think 'complements' is the correct word to use here.

**Responses to referees:**

**Referee #1:**

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First, they generate an inducible RNAi transgenic line in the ubp13 mutant background targeting UBP12. They perform physiological measurements of this line on regular medium and in the presence of brassinolide. They then use western blotting to track the levels of the BRI1 protein in this line in the presence of brassinolide or an inhibitor of vacuolar trafficking. They next perform protein interaction studies between UBP12 and BRI1 and ubiquitin and BRI1.Then they cross their inducible line into multiple lines expressing wild-type BRI1 or BRI1 with modifications to its ubiquitin status. Finally, they examine the subcellular localization of BRI1 in the RNAi inducible line.

From these experiments the authors conclude that UBP12 and UBP13 participate in brassinosteroid signaling through the prevention of ubiquitin mediated endocytosis and degradation of BRI1. They also conclude that UBP12 and UBP13 are able to cleave K63-linked polyubiquitin chains on BRI1 that has been previously shown to be mediated by the E3 ubiquitin ligase, PUB13 (also confirmed here). The complementary approaches and detailed analyses provide solid evidence for their conclusions. There a just a few minor concerns regarding the manuscript that are detailed below:

1. It was previously shown that the same UBP proteins interact with BZR1 (Wang et al. Molecular and Cellular Proteomics 2013). This work appears to be absent from mention in the manuscript but may indicate an additional role for UBPs in controlling BR signaling. This should at least be mentioned in the discussion including how this would relate to the work presented.

**OUR RESPONSE:** Thank you very much for your suggestion. We discussed this point in the Discussion part of the revised manuscript (line 332 - 338). It reads as

"Moreover, ubiquitination also mediates the BZR1 and BES1 degradation through 26S proteasome or selective autophagy (Nolan *et al*, 2019). An early proteomics analysis had revealed that UBP12 and UBP13 were BZR1-interacting proteins and proved the interaction between UBP12 and BZR1 in the nucleus of *N. benthamiana* (Wang *et al*, 2013). Whether UBP12 and UB13 deubiquitinate BZR1 to regulate the BR signaling awaits further research and validation."

2. The inducible RNAi transgenic line seems like a useful tool since the double knockout of ubp12 and ubp13 is not viable. That being said, there are additional genetic resources (stable RNAi, partial loss-of-function alleles) that may be useful to complement the studies here. One might worry the inducible system could introduce variable expression depending on the experimental conditions. This would be difficult to account for. It would be nice to see additional expression data showing that the RNAi is equally effective at suppressing UBP12 expression in all of the genetic backgrounds presented. Also, the brssinolide treatment experiments could be performed with other alleles to confirm the results presented.

**OUR RESPONSE:** We appreciate the comment on the importance of establishing the inducible RNAi transgenic lines. Indeed, we have experienced that the *UBP12* transcript level in the *ubp12/13* stable RNAi lines reported in (Jeong *et al*, 2017) was not reduced. Thus, we have generated the DEX inducible RNAi *ubp12/13* lines and observed the reduction of the *UBP12* expression level in all genotypes expressing the inducible *UBP12RNAi* construct upon DEX treatment. As shown in Appendix Fig S1B and S3A, two independent alleles showed the reduced expression of *UBP13* and *UBP12* upon DEX treatment compared to mock treatment. In addition, we have generated the *35S:UBP13* overexpression transgenic lines to validate the involvement of UBP12/13 in the BR signaling. In contrast to *ubp12i/ubp13* double mutants, two independent lines of *35S:UBP13* showed the hypersensitivity to BL (Appendix Fig S2A-C) (line 144 - 148), and this further supported our observation that UBP12 and UBP13 may positively regulate the BR signaling.

3. The dwarf phenotypes of the *ubp12i/ubp13* plants in the light are striking, similar to the *bri1* mutant, and are used to indicate that the BR pathway is compromised. This does not seem to be the case in the dark (Figure S3). While the light grown plants resemble the *bri1* mutant, the dark grown plants seem less severe than expected for a *bri1* mutant grown in the dark. Unfortunately, the bri1 mutant was not included as a control in the dark grown, brassinolide treatment, experiment. The *bri1* control and its responsiveness should have been included for reference in this experiment.

**OUR RESPONSE:** Thank you for this suggestion. We have included the *bri1*-null mutant in the dark-grown hypocotyl growth assay to examine their responsiveness to BL treatment (Appendix Fig S2B and C). As this referee expected, the *bri1*-null mutant grown in the dark displayed very short, thickened hypocotyls and fully opened cotyledons, and was completely insensitive to BL. Compared with the *bri1*-null mutant phenotype, the *ubp12i/ubp13* mutant phenotype was less severe, although the sensitivity to BL was reduced compared to that of wild type (Appendix Fig S2B-E).
The similar insensitive phenotype of *ubp12i/ubp13* mutant and *bri1*-null mutant in the BL rescue assay (Fig 1A), where BR responsiveness was assessed at a mature stage, implicated the possibility that UBP12 and UBP13 regulate the BRI1 stability in a growth stage-dependent manner. We have included the following sentences in the Discussion:
"At the seedling stage, *ubp12i/ubp13* plants had only a partially reduced BR sensitivity (Fig 1B-D, Appendix Fig S2), in contrast to the pronounced insensitive phenotype of the *ubp12i/ubp13* mature plants (Fig 1A), indicating that such regulation may also depend on the growth stage. " (line 366- 370).

**Referee #2:**

In their manuscript the authors address the role of two members of the Arabidopsis UBP deubiquitinase family in the regulation of BRI1 homeostasis.

BRI1 sorting and proteolytic turnover appears to be mediated by reversible poly-ubiquitination, involving activity of PUB E3 ubiquitin ligases. Here, the authors provide evidence for an antagonistic function for UBP12 and UBP13 mediating BRI1 deubiquitination and stabilization.

Several points support the authors' claim:

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This observation is accentuated further by quantification of mCitrine-tagged BRI1 and bri1-25KR reporter signals, revealing increased vacuolar targeting, specifically of wild type BRI1, when expressed in ubp12i/ubp13.

Overall, the data presented make a very strong case for a critical role of deubiquitinase activity in the modulation of plasma membrane protein activity in Arabidopsis. These findings add substantially to our understanding of ubiquitination-related signaling in higher plants.

**OUR RESPONSE**: We appreciate the accurate summary of our work and positive comments from this referee.

There is one thing the authors need to consider:

In Figure 3, the authors present their analysis of deubiquitination activity of UBP13 in vitro. I was intrigued by Fig 3D, suggesting PUB13 enzymatic activity in formation of K63-linked polyubiquitin chains. Whilst still not entirely resolved, some elements participating in K63-linked polyubiquitin chain formation appear fairly conserved in eukaryotes. RING-finger type E3 ligases seem to be involved as well as a unique type of E2 heterodimers composed of Ubc13 (UBC35/36 in Arabidopsis) and UEVs. Apart from that, additional E3 ligases have been implicated in K63-linked polyubiquitin chain formation, such as certain HECT proteins. There is also circumstantial evidence, suggesting that PUB13 might catalyze formation of such polyubiquitin chains, given its role in sorting control of plasma membrane protein. However, to my knowledge, no clear biochemical proof has been provided so far.

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**OUR RESPONSE:** We thank this referee for the insightful discussion and constructive comment. As suggested, we have performed *in vitro* ubiquitination assays using different ubiquitin K-to-R mutant variants. First, we demonstrated that the autoubiquitination activity of PUB13 formed both K48- and K63-linked polyubiquitin chains by *in vitro* ubiquitination assays using the ubiquitin KR variants, including K63R, K48R, K63-only, and K48-only (Appendix Fig S5A and B). Second, we confirmed that $BRI1_{CD}$ was modified with K63-linked polyubiquitin chains by PUB13 using the K63-linked chain-specific antibody (Appendix Fig S5C and D). In addition, by incubating MBP-$BRI1_{CD}$ and GST-PUB13 with the K63R ubiquitin variant, we detected a clear reduction of polyubiquitinated MBP-$BRI1_{CD}$ proteins compared with those incubated with wide-type or K48R ubiquitin (Appendix Fig S5D). Taken together, these data support that PUB13 can catalyze at least K63-ubiquitination to BRI1 *in vitro*. We added these data in the Result section in the revised manuscript (line 203 - 217).

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The work by Luo et al provides clear evidence for the involvement of two deubiquitinating enzymes, UBP12 and UBP13, in regulating brassinosteroid (BR) signaling via mutant analysis. The manuscript may be further improved by showing the effects of UBP12 and/or UBP13 overexpression on BRI1 ubiquitination and stability and BR response. Some outstanding questions include: how does brassinolide (BL) affect UBP12/13 association with BRI1? How does brassinolide affect UBP12/13 mediated deubiquitination of BRI1?

**OUR RESPONSE:** We appreciate this referee's comments on recognizing our main discovery of the involvement of UBP12 and UBP13 in regulating BR signaling. We also appreciate the constructive suggestion from this referee in terms of strengthening the effects of UBP12 and/or UBP13 overexpression on BRI1 ubiquitination and stability and BR response. Towards this end, as described below in our point-by-point responses, we have generated *35S:UBP13* transgenic plants and demonstrated that two independent *35S:UBP13* transgenic lines exhibited hypersensitivity to BL treatment compared to wild-type plants, which is in contrast to the reduced sensitivity to BL in *ubp12i/ubp13* mutants (Appendix Fig 2A-C). Additionally, we have compared the endogenous BRI1 protein levels in wild-type plants and *ubp12i/ubp13* mutants in the absence of protein synthesis by deploying the cycloheximide (CHX)-chase experiment (Appendix Fig S4). In the same time course experiment, the half-life of the BRI1 protein was clearly reduced to less than 5 hours in *ubp12i/ubp13* mutants compared to wild-type plants (Appendix Fig S4). To further characterize the function of UBP12/13 in BR response, we have performed additional experiments and compared BL-induced phenotypes in *ubp12i/ubp13* mutants and wild-type plants in the light (Fig 1B and C, Fig 4C). Compared to the wild-type plants, *ubp12i/ubp13* mutants showed reduced sensitivities to BL at all concentrations tested. We also introduced the $BRI1_{25KR}$ -mCit construct in *bri1* or *bri1/ubp12i/ubp13* mutants and examined the BR responses in the light (Fig 4C and Appendix S7A). In line with the observation that $BRI1_{25KR}$ impairs BRI1 ubiquitination (Martins *et al*, 2015), BL-induced hypocotyl phenotypes were similar for both *bri1* and *bri1/ubp12i/ubp13* transgenic plants expressing $BRI1_{25KR}$-mCit, which was in contrast to the BL-reduced hypocotyl elongation of *bri1/ubp12i/ubp13* mutant expressing BRI1-mCit when compared to those of BRI1-mCit in the *bri1* mutant (Fig 4C). At a mature growth stage, all $BRI1_{25KR}$-mCit expressing plants in *bri1* or *bri1/ubp12i/ubp13* mutant backgrounds were fully responsive to exogenous BL exhibiting long curled petioles, whereas BR responses of the *ubp12i/ubp13* mutants expressing BRI1-mCit were impaired (Appendix S7B). Taken together, by generating additional transgenic plants, monitoring the endogenous BRI1 protein level and plant response to BL in the light and dark, we have provided

genetic and biochemical data to further strengthen our observation on the critical role of UBP12 and UBP13 in regulating BRI1 ubiquitination and stability and BR responses.

We concur with this referee, and we are keen to investigate further whether BL may affect UBP12/13 association with BRI1 and its deubiquitination. It is worth noting that UBP13 contains a MATH domain, which presumably mediates the protein-protein interaction (Lee *et al*, 2019; Lindbäck *et al*, 2021). A unique feature of UBP protein family is likely to remove the substrate-bound proximal ubiquitin (Mevissen & Komander, 2017). It is plausible that the interaction between UBPs and their substrates is transient, thus it may be difficult to detect. This can be different from other DUBs, which remove ubiquitin from the distal side and associate with the substrates for a longer time. In line with these speculations, our multiple attempts for co-immunoprecipitation assays using either total proteins or microsomal proteins derived from protoplasts co-expressing UBP13 with BRI1, or from *35S:UBP13* (FLAG-tagged) transgenic plants with or without BL treatment failed to reach a conclusion whether BL affects UBP13 association with BRI1. It is also possible that UBP12 and UBP13 play roles in regulating both basal and ligand-activated BRI1 since BRI1 has been shown to be ubiquitinated in the presence of brassinazole (BRZ, a BR biosynthetic inhibitor) (Martins *et al*, 2015). Future efforts on generating stable transgenic plants carrying BRI1 and UBP13 under the control of their native promoters with a pretreatment of BRZ treatment followed by BL treatment might help to address whether and how BL may regulate UBP12/13 association and deubiquitinating of BRI1. We hope that this referee could agree with us this as an interesting perspective to follow in the future and beyond the main theme of this work.

Further points to consider:

Figure 1B - How does hypocotyl length of the ubp mutants compare to bri1 mutants +/- BL? What about the effect of BL on hypocotyl length of light grown mutant seedlings compared to WT? The amount of BL used in some assays (500/1000) seems high.

**OUR RESPONSE:** As suggested by this referee, in the revised manuscript, we have

included the *bri1*-null mutant alongside with *ubp12i/ubp13* double mutants in BL responsive hypocotyl growth assays in the dark condition. In addition, we also included two independent *35S:UBP13* transgenic lines in these assays. As shown in Appendix Fig S2B and C, the *ubp12i/ubp13* plants showed reduced sensitivities to exogenous BL but not to the same extent as the *bri1*-null mutant, opposed to the BR hypersensitivity of *35S:UBP13* plants. Under light condition, both lines of *ubp12i/ubp13* double mutants showed significantly reduced sensitivities to BL compared to the wild-type plants at all concentrations tested (10 to 1000 nM BL) (Fig 1B and C). (line 132-135). Regarding the referee's concern about BL concentration, we tested the BR-dose dependent responsiveness of *ubp12i/ubp13* double mutants in both light and dark, using BL concentrations ranged from 10 nM to 1000 nM (Fig 1B and C, Appendix Fig S2D and E). 1000 nM BL was also routinely used in BR rescue assays as in Fig 1A and Appendix Fig S7B. We have observed differences in the potency of brassinolide depending on the product, manufacture and dilution solvent *per se*. For instance, in *Zhou* et al., 2018, *PNAS*, 50 nM BL treatment increased the hypocotyl length in wild-type plants to > 250% (compared to DMSO mock) in the light. However, under our experimental condition, 100 nM BL (Cayman) treatment could only increase the hypocotyl length to ~ 200% (compared to EtOH mock). Therefore, we have used 500/1000 nM BL (Cayman) in our assays mentioned above. In line with this, follows are examples using up to 1000 nM BL to examine BR responses (Oh *et al,* 2012; Zhu *et al*, 2017; Hou *et al*, 2019; Zhang *et al*, 2021).

Figure 1 - The level of dephosphorylated BES1 in *ubp12i/ubp13* mutant #1-7 (fig 1D) is not significantly different from WT in the presence of BL.
However, the response of #1-7 to BL is significantly different from that of wild type (fig 1B). While ubp12i/ubp13 mutant #12-8 has significantly less dephosphorylated BES1 but the BL response is not as drastic as shown for #1-7. How is this explained?

**OUR RESPONSE:** We agree with the referee's remark. Although both *ubp12i/ubp13* mutant lines, #1-7 and #12-8, showed similar trends there was some variation due to

likely chemical treatments and tissue sampling. To overcome this, we have repeated the BES1 dephosphorylation experiment with an increased number of biological replicates (n= 6) under the same experimental condition. As a result, both lines of *ubp12i/ubp13* mutants showed statistically significant less dephosphorylated BES1 proteins upon BL treatment compared with wild-type plants (Fig 1E, $p<0.01$ evaluated by one-way ANOVA and post-hoc Tukey's test). Considering the different growth conditions between BES1 dephosphorylation experiment in the light and BL-induced hypocotyl phenotypes in dark, the responsiveness of *ubp12i/ubp13* to BR may not be simply compared. Therefore, we investigated BR response in light-grown plants by measuring the hypocotyl elongation. Both lines of *ubp12i/ubp13* mutants showed reduced hypocotyl elongation at all BL concentrations tested (Fig 1B and C, $^{**}p<0.01$ evaluated by one-way ANOVA and post-hoc Tukey's test). This result is in agreement with the accumulation of dephosphorylated BES1 in those lines in response to BL.

Figure 1C - identify phosphorylated and unphosphorylated BES1 on anti-BES1 blot.

**OUR RESPONSE:** We have labeled these bands in the revised Figure as suggested (Fig 1D).

Figure 2A - bands requires quantification, compare BRI1 levels in mutant to WT.

**OUR RESPONSE:** We quantified the band intensity (Fig 2A).

Figure 2 - Is it possible to look at the abundance/stability of BRI1 in the absence of protein synthesis?

**OUR RESPONSE:** As suggested by the referee, we have performed a cycloheximide (CHX)-chase experiment to assess the BRI1 protein stability in the absence of protein synthesis (Appendix Fig S4). The endogenous BRI1 proteins have a half-life around 5 h in wild-type plants similar to previously reported (Geldner *et al*, 2007). Importantly,

in *ubp12i/ubp13* plants, the half-life of BRI1 had reduced to less than 5 h. The BRI1 protein level decreased to 59% in wild-type plants and to 39% or 44% in *ubp12i/ubp13* plants five hours after CHX treatment. These results corroborate the role of UBP12 and UBP13 in the maintenance of the BRI1 protein stability. We included the new data in revised manuscript (line 163 - 167).

Figure 3D and E - Demonstrate deubiquitination in vivo by overexpressing UBP12/13 or at least 'semi' in vivo - isolate BRI1 from plants and use as substrate for deubiquitination.

**OUR RESPONSE:** As suggested by the referee, we performed the "semi" *in vivo* deubiquitination assays and included the new data in Appendix Fig S6. We first immuno-precipitated BRI1-mCitrine proteins from BRI1-mCit/*bri1* transgenic plants and then co-incubated with GST-UBP13 or GST-UBP13$^{C207S}$ recombinant proteins. The abundance of polyubiquitinated BRI1-mCitrine was reduced by incubating with UBP13 compared to that with UBP13$^{C207S}$, which is a deubiquitinating-inactive mutant. The data support that UBP13 possesses the *in vitro* DUB activity against ubiquitinated BRI1 *in planta*.

Figure 4 - what is the response of the transgenic plants to BL compared to WT? BRI125KR-mCit/bri1/ubp12i/ubp13 seems to be smaller and have less lateral roots compared to BRI125KR-mCit/bri1.Is it possible to quantify size/root growth.

**OUR RESPONSE:** Thank you for the useful suggestion. We subjected the *bri1* and *bri1/ubp12iubp13* mutants expressing either BRI1-mCit or BRI1$_{25KR}$-mCit to exogenous BL to study their BR responsiveness (Fig 4C, Appendix Fig S7A). In the hypocotyl growth assay, loss of UBP12 and UBP13 suppressed the hypocotyl elongation promoted by exogenous BL compared to corresponding lines expressing wild-type BRI1-mCit. Whereas the responsiveness to BRs, in terms of BL-induced hypocotyl elongation in the light, was not affected in *ubp12i/ubp13* plants expressing

BRI1$_{25KR}$-mCit (Fig 4C, Appendix Fig S7A). Similarly, exogenous BL did not restore the dwarf phenotype of BRI1-mCit/*bri1/ubp12i/ubp13* plants, and did not or slightly induced the petiole bending in those lines. On the contrary, BRI1$_{25KR}$-mCit/*bri1/ubp12i/ubp13* plants remained fully responsive to BL and exhibited long, curled petioles, as BRI1$_{25KR}$-mCit/*bri1* plants did (Appendix Fig S7B). We also quantified root growth by measuring primary root length and lateral root density in the transgenic plants (Appendix Fig S3B). Both primary root growth and lateral root development were impaired in *ubp12i/ubp13* background. The expression of BRI1$_{25KR}$-mCit partially rescued the root growth defects (line 249， 258， 260 - 268).

Figure 5A - The difference in fluorescence intensity between WT and *ubp12i/ubp13* is not clear. Compare to WT, *ubp12i/ubp13* is expected to show less plasma membrane localized BRI1 due to increase internalization and degradation, this is not apparent in the figures. Further assays are needed to better demonstrate increased vacuolar targeting.

**OUR RESPONSE:** The vacuolar targeting of BRI1-mCit was measured as the relative PM/intracellular fluorescence intensity in the images presented in Fig 5A and it was slightly but significantly increased in the two *ubp12i/ubp13* lines when compared to the wild type UBP12/UBP13 (Fig 5B). In contrast, the BRI1$_{25KR}$–mCit localization was not affected in the *ubp12i/ubp13* mutant (Fig 5C). Because those are different transgenic lines and different plants we cannot simply compare the PM intensity of BRI1-mCit. In order to improve the visibility, we converted the images' LUT to multicolored LUT (Fig 5A and C).

In addition, we further analyzed the vacuolar targeting of BRI1 by AFCS uptake analysis in wild type and *ubp12i/ubp13* plants. AFCS is a bioactive fluorescent BR which allows us to visualize the endocytosis of BR-BRI1 complexes. Results showed that vacuolar accumulation of AFCS signal was strikingly promoted in *ubp12i/ubp13* plants compared with the wild-type plants, suggesting that UBP12 and UBP13 negatively regulate BR-BRI1 complexes internalization and vacuolar targeting (Fig 5E

and F). Increased AFCS uptake in *ubp12i/ubp13* plants corroborates our observations that BRI1-mCit vacuolar targeting is enhanced in the *ubp12i/ubp13* mutant (line 293 – 298).

Some minor edits:
Line 50-51, requires editing for clarity.

**OUR RESPONSE:** We have modified it that reads as follows “The versatility of ubiquitin-dependent signaling is imposed by the diversity of polyubiquitin chains, that is, the substrate-attached ubiquitin can be further ubiquitinated on its seven internal lysine (K) residues or on the N-terminus encompassing complex topologies.” (line 50 - 53).

Line 52-54, requires references.
**OUR RESPONSE:** We have added the reference (Oh *et al*, 2018) (line 56).

Line 55, "The antagonistic process of ubiquitination" should read (?) "The antagonistic process to ubiquitination" or "The antagonistic process of deubiquitination"

**OUR RESPONSE:** We have revised it as suggested. Now it is “The antagonistic process to ubiquitination” (line 57).

Line 63, Ewan et al, 2011 demonstrated deubiquitinating activity for UBP12 (not UBP13).

**OUR RESPONSE:** We thank this referee to point out this. We have revised it as suggested.

Line 124, "the most active BR, complements the dwarf phenotype of ubp12i/ubp13." I don't think 'complements' is the correct word to use here.

**OUR RESPONSE:** We agree with the referee's comment and replaced "complements" with "restores".

**References:**


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Dear Dr. Sato

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 very positive about the study and request only minor changes to clarify text and figures.

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

1) Your study will be published as a short report. For short reports, the revised manuscript should not exceed 27,000 characters and the results and discussion sections must be combined.

2) Appendix: please add page numbers to the table of content and please remove the Appendix figure legends from the main manuscript file.

3) Please change the heading of "Competing Interest Statement" to "Conflict of Interest".

4) Please specify the contribution of the co-author Libo Shan's in the respective field in the online submission system.

5) Please add callouts to Appendix Figs S4 and S6 wherever appropriate.

6) Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return the revised file with tracked changes with your final manuscript submission. I have also taken the liberty to make some changes to the title and abstract that I ask you to review.

7) Could you please also review the minor changes I introduced to the summary text?

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

With kind regards,

Martina Rembold, PhD
Senior Editor
EMBO reports

**********

Referee #1:

They have adequately addressed all of my concerns.

Referee #2:

All my earlier concerns have been addressed adequately.

Referee #3:

The revised manuscript is greatly improved, and the authors adequately addressed the reviewers concerns. Issue to consider: Figure S6 - I suggest to the authors that the experiment to demonstrate in in vivo deubiquitination of BRI1 was not very successful. This is the level of BRI1 ubiquitination in the presence and absence of a functional UBP13 is quite similar suggesting limited to no deubiquitination of BRI1. Regardless, ample evidence is provided to support a link between UBP13 and BRI1 regulation. Figure S4 - suggest comparing BRI1 protein levels in CHX treated ubp mutant to the zero-time point for each genotype and not to that of WT.

January 24, 2022
Dr. Martina Rembold
Senior Editor, *EMBO reports*

Manuscript #: EMBOR-2021- 53354V3

Dear Dr. Rembold:

Enclosed please find our revised manuscript entitled "Deubiquitinating enzymes UBP12 and UBP13 stabilize the brassinosteroid receptor BRI1" that we would like to resubmit for consideration for publication in the *EMBO reports* as a Scientific Report.

Thank you very much for your positive reply and the kind feedback regarding our manuscript. We modified our manuscript as suggested. A point-by-point response to each of the comments is attached below.

We appreciate your time and effort in handling our manuscript and hope that the revised manuscript is now suitable for publication in *EMBO reports*.

Sincerely yours,

Takeo Sato
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**Reply to Editor's and Reviewers' comments**

**Editor's comments**

1) *Your study will be published as a short report. For short reports, the revised manuscript should not exceed 27,000 characters and the results and discussion sections must be combined.*

OUR RESPONSE: We have reformatted our manuscript as suggested.

2) *Appendix: please add page numbers to the table of content and please remove the Appendix figure legends from the main manuscript file.*

OUR RESPONSE: We removed the figure legends for the Appendix and added page numbers to the content table as requested.

3) *Please change the heading of "Competing Interest Statement" to "Conflict of Interest".*

OUR RESPONSE: We have changed the heading in the manuscript.

4) *Please specify the contribution of the co-author Libo Shan's in the respective field in the online submission system.*

OUR RESPONSE: We specified her contribution in the online submission system.

5) *Please add callouts to Appendix Figs S4 and S6 wherever appropriate.*

OUR RESPONSE: We have clarified the figure legends and re-labeled the figures. In Appendix Fig S4A, we indicated the band intensity relative to the zero-time point in respective genotypes and clarified the figure legend as. In Appendix Fig S4B, we indicated the biological replicates. In Appendix Fig S6B, the Y axis label has been changed to "Ratio of Ubn-BRI1/BRI1", and quantification method has been clarified in the legend. Also, we have modified manuscript to clarify this point (line 246-248).

6) *Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return the revised file with tracked changes with your final manuscript submission. I have also taken the liberty to make some changes to the title and abstract that I ask you to review.*

OUR RESPONSE: We have clarified the points asked by production/data editors, details are described in 'Our response 5' as well as in the main text. Thank you for your modifications on the title and abstract, we agree with the changes. Additionally, we changed a description "Ubiquitin-deficient" to "Ubiquitination-deficient" through the

manuscript. All the changes are recoded with track-change tool and highlighted by green color.

*7) Could you please also review the minor changes I introduced to the summary text?*

OUR RESPONSE: Thank you for the modification, we agree with the changes.

**Reviewer #3's comments**

*The revised manuscript is greatly improved, and the authors adequately addressed the reviewers concerns. Issue to consider: Figure S6 - I suggest to the authors that the experiment to demonstrate in in vivo deubiquitination of BRI1 was not very successful. This is the level of BRI1 ubiquitination in the presence and absence of a functional UBP13 is quite similar suggesting limited to no deubiquitination of BRI1. Regardless, ample evidence is provided to support a link between UBP13 and BRI1 regulation.*
*Figure S4 - suggest comparing BRI1 protein levels in CHX treated ubp mutant to the zero-time point for each genotype and not to that of WT.*

OUR RESPONSE: We thank the reviewer for the positive reply and kind feedback to our revision manuscript. Regarding to Fig S6, it's important to quantify the ubiquitinated BRI1 amounts relative to basal (intact) BRI1 protein amounts. Although the levels of ubiquitinated BRI1 on the P4D1 blot did not differ much between samples incubated with native UBP13 and with UBP13$^{C207S}$, the basal BRI1 amount was higher in native UBP13 samples. As a result of the quantification, ratio of ubiquitinated BRI1/basal BRI1 is different between the samples incubated with native UBP13 and with UBP13$^{C207S}$ (Fig. S6B). To clarify this point, we modified the label of the graph as well as the Figure legend for Fig. S6. Also, we have modified manuscript to clarify this point (line 246-248).
In response to Fig S4, we thank the reviewer for the constructive suggestion, and we have modified the band intensity labels in S4A to compare BRI1 protein levels in CHX treated *ubp12i/ubp13* to the zero-time point for each line.

Dr. Takeo Sato
Hokkaido University
Faculty of Science
Sapporo 060-0810
Japan

Dear Dr. Sato,

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

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Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Martina Rembold, PhD
Senior Editor
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## 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.

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### 1. Data

**The data shown in figures should satisfy the following conditions:**

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- 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.

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
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  - are tests one-sided or two-sided?
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  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

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| 1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size? | In this study,numbers of biological replicates and Arabidopsis seedling used in each experiment have been given in the Figure legends. |
| 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? | No samples have been excluded from our analysis. |
| 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. | Yes, one-way ANOVA and post-hoc Tukey's test was used to analyze significant test of difference. |
| Is there an estimate of variation within each group of data? | Yes, this is provided through the use of box and whisker plots or standard deviation of the mean. |

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| Is the variance similar between the groups that are being statistically compared? | Yes. |

## C- Reagents

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| 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 catalog numbers or citation of antibodies are included in the Materials and Methods section. |
| 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | NA |

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

## D- Animal Models

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| 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

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| 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

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| 18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions | NA |
| 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

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| 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. |