EMBO reports

TIFA has dual functions in *Helicobacter pylori*-induced classical and alternative NF- B pathways

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1st Editorial Decision 9th Apr 2021

Dear Dr. Naumann,

Thank you for the submission of your research manuscript to EMBO reports. We have now received the reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, the referees think that these findings are of interest. However, they have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and I feel all their points need to be addressed, I will not detail them here.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript or in the detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and we have therefore extended our 'scooping protection policy' to cover the period required for full revision. Please contact me to discuss the revision should you need additional time, and also if you see a paper with related content published elsewhere.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

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

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The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs

to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

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

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

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5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an appropriate public database. If no primary datasets have been deposited, please also state this a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

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Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

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

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at:
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Finally, please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript. Please find instructions on how to link the ORCID ID to the account in our manuscript tracking system in our Author guidelines:
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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

Referee #1:

This manuscript addresses an interesting feature of the colonizing human pathogen H. pylori, which directs, via the effector molecule ADP-heptose, the activation of ALPK1 and TIFA. Here the authors identified for the first time that TIFA activates non-canonical NF-kB signaling via binding to TRAF2 and canonical NF-kB signaling via binding to TRAF6. Thus, H. pylori infection executes the activation of both NF-kB pathways at the same time via two different TIFAsomes.
The reviewer has the following suggestions to improve the manuscript:

1) Fig. 1B: The phosphorylation of TAK1 was revealed by a phospho-specific antibody, please also show expression of unmodified TAK1. This ensures that changes seen by the phospho-specific antibody do not reflect changes in protein amount.

2) TIFA binds to TRAF2 and TRAF6 (Fig. 2C), thus, the observation of two TIFAsomes is interesting. To corroborate the data of two independent TIFAsomes it will be valuable to see whether the knockdown of TRAF2 (with relevance for non-canonical NF-kB) affects the classical NF-kB pathway.

3) Along the line of the previous point, the data could be further strengthened by the demonstration that TIFA binds directly to TRAF2 (and the NIK regulating complex - TRAF2/TRAF3/ciAP1 - per se) using knockdown of cIAP1 or TRAF3 in a TIFA coIP.

4) H. pylori-dependent cIAP1 degradation (Fig 3D) as the base for non-canonical NF-kB could be supported by showing, in an immunoprecipitation of cIAP1, that cIAP1 is K48 ubiquitinated. This would further support the data of MG132 treated cells (EV3B).

5) Please show in all Western blots the position of at least one MW marker.

6) The conclusion part in the manuscript could be slightly extended by a statement which cellular consequences could be associated with the dual activation of both NF-kB pathways in H. pylori infection

7) It might be interesting for the broader readership to include into the introduction more information on how the ADP-heptose is translocated from the colonizing H pylori to the eukaryotic cells and whether it is a virulence factor?

Referee #2:

Maubach and colleagues have examined the TIFAsome-dependent activation of the classical and alternative NfKB pathways in response to infection with H. pylori. The authors used in vitro models to probe signaling complex assembly downstream of TIFAsome activation through western bloting, immunoprecipitation and immunofluorescence to identify key components of the classical NFKB pathway (TRAF6, TAK1) and alternative (noncanonical) NFKB pathway (TRAF2/3, cIAP1) as interacting partners with TIFA. The also showed that this interaction depends on ALPK1 and its ligand ADP-Heptose, and further, this interaction leads to the accumulation of NIK and phospho-p100 of the alternative NFKB pathway. Based on these data the authors conclude that TIFAsome activation initiates alternative NFKB pathway signaling. While the experiments presented here are mostly well conducted, these findings don't greatly advance the fields of Helicobacter or TIFA

biology. TRAF2 (linked to the noncanonical pathways) was previously described as a TIFA interacting partner (1). The novel aspects of this manuscript are the interactions of TIFA with TRAF3 and the accumulation of NIK and p-p100 because of H. pylori infection. However, given the data known from other systems, this is not surprising. As detailed below there are some concerns that should be addressed to make some of the conclusions more convincing.

Moreover, the authors completely rely on cancer cell lines and makes no attempt to validate their conclusions in more relevant infection models. Additionally, identifying TIFA-dependent activation of the alternative NFkB pathway doesn't provide any insight to the relevance of this pathway to the infection. Simple knockout/knockdown experiments coupled with even basic growth and survival measurements could have provided the reader with a clearer idea of the importance of the molecular findings. Finally, TIFAsome activation has been reported as a very early response to H. pylori and additional mechanisms of NFkB activation overlap to encompass an intricate inflammatory response with rapid signaling kinetics. This work recapitulates the fast activation kinetics of the TIFAsome, but this model (very high MOI, short time course) leaves the physiological relevance of this interaction as an open question. TIFAsome activation during H. pylori infection may be an important host response but the complexity of this receptor interaction is poorly understood. Therefore, shedding light on the molecular interactions and signaling pathways activated by H. pylori is a valuable addition to the field. This work is a step in the right direction but at present has limited impact.

Major concerns:
1. Fig 1E is misinterpreted in the text page 4 line 125. They did see accumulation of phosphor-p100 and p-IkBa, just not as much
2. Fig2a - the nonspecific band accumulates. Could this band be something relevant, like a different modification?
3. Figure 3A, 3C, and 3D - The TRAF2 and TIFA interaction kinetics don't agree. In 3A there is a maximum at 30 minutes and almost nothing at 2 hours. In C, the TRAF2/TIFA interaction gives a strong band at 2.5 hours. In D there a maximum intensity band at 1.5 hours and a very strong band at 2.5 hours implying that a 2 hours timepoint would still have a strong band. Since TIFA/TRAF2 interaction kinetics may determine the strength of alternative NfKB pathway activation please comment on these seemingly contradictory kinetics.
4. 3E is very hard to interpret. The E178A mutant has much less accumulation of NIK in comparison to WT. Why is the if E178 so much weaker if it is important for TRAF6 binding, but not TRAF2? The oligomerization in 3F also looks reduced in comparison to WT. The model described in lines 197-204 may be inadequate.
5. Figure 4 - TIFA-tdTomato doesn't look the same in any panel. Especially E. Also, in extended figures 4 and 5 TIFA expression is extremely different between the cells and some cells have undetectable expression in these images. From these images it would be impossible to reproduce the quantitative analysis.
6. The gmhA knockout experiments need further controls. First, experiments using a knockout strain need complementation to address possible polar effects or other genetic interactions. Second, gmhA is a key gene in the LPS biosynthetic pathway. How sick is this strain? An alternative explanation for the observations in figure 1b and 1c is that the knockout strain has growth and virulence defects from perturbations of the outer membrane. In support of this, knockout of gmhA and gmhB seem to have a deficiency in CagA translocation in comparison to an isogenic WT strain. This contributes to a strong reduction in IL-8 secretion despite equivalent levels of CagA expression (2).

1. Fu J, Huang D, Yuan F, Xie N, Li Q, Sun X, Zhou X, Li G, Tong T, Zhang Y. 2018. TRAF-interacting

protein with forkhead-associated domain (TIFA) transduces DNA damage-induced activation of NF-κB. J Biol Chem 293:7268-7280.
2. Pfannkuch L, Hurwitz R, Traulsen J, Sigulla J, Poeschke M, Matzner L, Kosma P, Schmid M, Meyer TF. 2019. ADP heptose, a novel pathogen-associated molecular pattern identified in Helicobacter pylori. FASEB J fj.201802555R.

Referee #3:

The authors have investigated the role of TIFA in NF-kB signaling in epithelial cells to the bacterial pathogen, Helicobacter pylori. The work presents two major claims. First, it is claimed that H. pylori induction of "TIFasome" formation with the TIFA-interacting partner, TRAF6, leads to the activation of classical NF-kB signaling. The second claim is that TIFA interacts with TRAF2, resulting in proteasomal degradation of cIAP1 and activation of the alternative NF-kB signaling pathway. Although the first claim is not novel within the broad field of TIFA signaling, it does provide new information regarding the H. pylori->TIFA->NF-kB signaling axis. There are currently seven articles (including two reviews) on TIFA and H. pylori in Pubmed, while virtually nothing is known regarding the pathophysiological role of TIFA in H. pylori disease in vivo. The current work should provide impetus for research in this regard. The second claim is of higher novelty and likely to be of broad interest to researchers studying alternative NF-kB signaling.

The data are nicely presented, are of very high quality and consistent with the conclusions drawn. The manuscript is exceptionally well written. The authors have adequately discussed their findings in light of the existing literature, though a couple of important references have been omitted (see comments below). Overall, the manuscript presents new data that are likely to be of interest to those in both the H. pylori field and, more broadly, to those studying NF-kB signaling in response to microbial pathogens. Nevertheless, some additional controls are needed to confirm specificity of the observed phenomena to H. pylori activation of TIFA signaling. Specific points for the authors' consideration are listed below.

Major points:
1) There is no indication regarding the numbers of independent experiments that were performed. This information needs to be supplied for each data set.
2) The authors have confirmed their key findings for AGS cells using multiple cell lines and clones, but have only used one H. pylori strain (P1). As reported previously (Gall et al. doi.org/10.1128/mBio.01168-17), there are significant strain-specific differences in the genes encoding key steps in LPS biosynthesis. These differences may impact ALPK1/TIFA signaling. It is therefore important that the authors test at least an additional pair of H. pylori WT and HP0587 mutant strains, or more than one WT strain, on the AGS, AGS ALPK1-KO and AGS TIFA-KO cell lines (as per Fig. 2A). This will confirm that H. pylori activation of the alternative NF-kB signaling pathway is not a strain-specific phenomenon.
3) TIFA was shown to interact with TRAF6 and TRAF2 by over-expression using TIFA-KO cells expressing His-tagged recombinant TIFA protein. Although this has been described in other models, there are always concerns regarding over-expression as the sole approach to studying protein interactions. Can endogenous TIFA be pulled down in AGS WT cells stimulated with P1? The anti-TIFA antibody seems of good quality and endogenous TIFA levels seem sufficiently high to be able to do this. Another approach would be to perform the experiment with TIFA-KO cells expressing His-tagged recombinant TIFA, but use HP0587 mutant bacteria.
4) The transfection work with TIFA-dtTomato in AGS-TIFA KO cells (Fig. 4) needs a control. It is not

entirely clear, but seems that the observed co-localization of TIFA, TRAF6, TRAF2 etc was in response to H. pylori stimulation (lines 205-208). Nevertheless, TIFA can self-oligomerize (line 85), so it is possible that over-expression may be sufficient to induce TIFAsome formation. The authors need to show TIFAsome formation in the absence of H. pylori stimulation and/or in response to the HP0587 mutant.

Minor points:
5) TIFA interactions with TRAF2 and TRAF6 have been reported previously (Kanamori et al. BBRC 290, 1108; Takatsuna et al. doi: 10.1074/jbc.M300720200), so it is not possible to claim "...TRAF6 and TRAF2 as NOVEL binding partners of TIFA.." (lines 37-38).
6) The Ohmae paper (line 55) described H. pylori activation of the alternative pathway in B lymphocytes and not epithelial cells. It is important to stress this detail in the text. Also, in contrast to the current work, Ohmae reported that H. pylori was unable to induce the alternative pathway in AGS cells. This difference should be discussed.
7) The authors need to cite the work of Hirata (DOI: 10.4049/jimmunol.176.6.3796). Those authors first reported the roles of TRAF6 and TAK1 in H. pylori induction of NF-kB signaling in epithelial cells. They also described that this signaling was MyD88-dependent. Do the authors have any data regarding the role of MyD88 in the TIFA-dependent responses observed here?
8) The work of Gall et al (mentioned above) should be cited.
9) Does TIFA lacking an FHA domain, needed for interactions with TRAF2/TRAF6, have any effect on NF-kB signaling?
10) The HP0858 gene codes for the enzymatic step after HP0587 in the LPS biosynthesis pathway and is the step responsible for the production of the TIFA substrate, HBP. Does the HP0585 mutant induce the same responses in AGS cells as the HP0587 mutant?
11) It is unclear why the amounts of the TIFA in this pulldown (Fig. 2E, line 162) are much lower when compared with the other blots.

Point-by-point response to the reviewers' reports

We thank the reviewers' for their valuable comments and suggestions. We have addressed the comments by additional experiments where appropriate and have revised the figures accordingly. We have also revised the manuscript accordingly and included some minor changes to improve clarity and language.

Reviewer #1:
This manuscript addresses an interesting feature of the colonizing human pathogen H. pylori, which directs, via the effector molecule ADP-heptose, the activation of ALPK1 and TIFA. Here the authors identified for the first time that TIFA activates non-canonical NF-kB signaling via binding to TRAF2 and canonical NF-kB signaling via binding to TRAF6. Thus, H. pylori infection executes the activation of both NF-kB pathways at the same time via two different TIFAsomes. The reviewer has the following suggestions to improve the manuscript:

1) Fig. 1B: The phosphorylation of TAK1 was revealed by a phospho-specific antibody, please also show expression of unmodified TAK1. This ensures that changes seen by the phospho-specific antibody do not reflect changes in protein amount.

We have included the immunoblot for TAK1.

2) TIFA binds to TRAF2 and TRAF6 (Fig. 2C), thus, the observation of two TIFAsomes is interesting. To corroborate the data of two independent TIFAsomes it will be valuable to see whether the knockdown of TRAF2 (with relevance for non-canonical NF-kB) affects the classical NF-kB pathway.

We have performed a knockdown of TRAF2 or TRAF6 (new Fig. EV2G and H). The following text was included in the manuscript (line 177) "*To elucidate the potential impact of TRAF2 and TRAF6 on the activation of H. pylori-induced classical and alternative NF-κB pathways, respectively, we performed a knockdown of TRAF2 or TRAF6. Knockdown of TRAF2 did not affect the activation of the classical NF-κB (Fig. EV2G), whereas the depletion of TRAF6 significantly inhibited the activation of the classical NF-κB but had no effect on the activation of the alternative NF-κB (Fig. EV2H). This suggests a distinct separation of the classical and alternative NF-κB pathways upon H. pylori infection on the level of TRAF6 and TRAF2, respectively."*

3) Along the line of the previous point, the data could be further strengthened by the demonstration that TIFA binds directly to TRAF2 (and the NIK regulating complex - TRAF2/TRAF3/ciAP1 - per se) using knockdown of cIAP1 or TRAF3 in a TIFA co-IP.

We have performed a knockdown of cIAP1 and a co-IP using TIFA antibody (new Fig. 3E). The following text was included in the manuscript (line 223) *"The transient interaction of TIFA with the NIK regulatory complex however is not directly mediated by cIAP1 because in cIAP1-depleted cells, we still found the transient, albeit enhanced, binding of TIFA to TRAF2 and TRAF3 (Fig. 3E). Interestingly, we also observed an inducible decline of TRAF2 in the lysates of cIAP1-depleted cells (Fig. 3E)"*

4) H. pylori-dependent cIAP1 degradation (Fig 3D) as the base for non-canonical NF-kB could be supported by showing, in an immunoprecipitation of cIAP1, that cIAP1 is K48 ubiquitinated. This would further support the data of MG132 treated cells (EV3B).

We have performed an IP of cIAP1 under denaturing conditions and subjected the eluates to immunoblot detection using a K48-linked ubiquitin-specific antibody (new Fig. EV3C). The text was revised accordingly (line 220): *"The proteasome-dependent turnover of cIAP1 was corroborated by the detection of K48-linked ubiquitinylation of cIAP1 upon H. pylori infection (Fig. EV3C)."*

5) Please show in all Western blots the position of at least one MW marker.

We have included MW marker throughout the figures.

6) The conclusion part in the manuscript could be slightly extended by a statement which cellular consequences could be associated with the dual activation of both NF-kB pathways in H. pylori infection

The following text was included in the manuscript (Line 251): "*This dual activation of both NF-κB pathways leads to complex gene regulation that could trigger a variety of cellular events including not only the induction of inflammatory cytokines by the classical NF-κB pathway, but also other physiological changes leading to gastric pathologies due to the alternative NF-κB pathway (Merga et al, 2016).*"

7) It might be interesting for the broader readership to include into the introduction more information on how the ADP-heptose is translocated from the colonizing H pylori to the eukaryotic cells and whether it is a virulence factor?

We have revised the text in the manuscript (Line 80): *"However, little is known about its uptake into the cytosol of cells. The uptake of heptose-1,7-bisphosphate (HBP), a precursor of ADP-heptose, is compromised by dynamin inhibition (Gaudet et al, 2015) and T4SS deficiency (Zimmermann et al, 2017). Exogenously added ADP-heptose, even without membrane permeabilization, has been shown to trigger NF-κB activation (Pfannkuch et al, 2019). Interestingly, bacteria without a T3SS, like enterotoxic Escherichia coli and Burkholderia cenocepacia, are also able to elicit ADP-heptose-dependent NF-κB activation in epithelial cells (Zhou et al, 2018)."*

Reviewer #2:

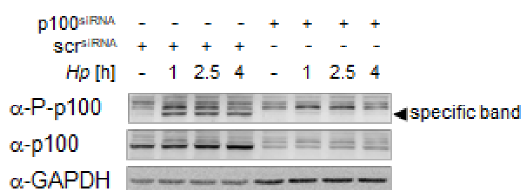
Maubach and colleagues have examined the TIFAsome-dependent activation of the classical and alternative NfKB pathways in response to infection with H. pylori. The authors used in vitro models to probe signaling complex assembly downstream of TIFAsome activation through western bloting, immunoprecipitation and immunofluorescence to identify key components of the classical NFKB pathway (TRAF6, TAK1) and alternative (noncanonical) NFKB pathway (TRAF2/3, cIAP1) as interacting partners with TIFA. The also showed that this interaction depends on ALPK1 and its ligand ADP-Heptose, and further, this interaction leads to the accumulation of NIK and phospho-p100 of the alternative NFKB pathway. Based on these data the authors conclude that TIFAsome activation initiates alternative NFKB pathway signaling. While the experiments presented here are mostly well conducted, these findings don't greatly advance the fields of Helicobacter or TIFA biology. TRAF2 (linked to the noncanonical pathways) was previously described as a TIFA interacting partner (1). The novel aspects of this manuscript are the interactions of TIFA with TRAF3 and the accumulation of NIK and p-p100 because of H. pylori infection. However, given the data known from other systems, this is not surprising. As detailed below there are some concerns that should be addressed to make some of the conclusions more convincing.
Moreover, the authors completely rely on cancer cell lines and makes no attempt to validate their conclusions in more relevant infection models. Additionally, identifying TIFA-dependent activation of the alternative NFkB pathway doesn't provide any insight to the relevance of this pathway to the infection. Simple knockout/knockdown experiments coupled with even basic growth and survival measurements could have provided the reader with a clearer idea of the importance of the molecular findings. Finally, TIFAsome activation has been reported as a very early response to H. pylori and additional mechanisms of NFkB activation overlap to encompass an intricate inflammatory response with rapid signaling kinetics. This work recapitulates the fast activation kinetics of the TIFAsome, but this model (very high MOI, short time course) leaves the physiological relevance of this interaction as an open question. TIFAsome activation during H. pylori infection may be an important host response but the complexity of this receptor interaction is poorly understood. Therefore, shedding light on the molecular interactions and signaling pathways activated by H. pylori is a valuable addition to the field. This work is a step in the right direction but at present has limited impact.

Major concerns:
1. Fig 1E is misinterpreted in the text page 4 line 125. They did see accumulation of phosphor-p100 and p-IkBa, just not as much

We have revised the sentence as follows (line 142): "In cells pre-incubated with Dynasore followed by either infection with H. pylori or treatment with exogenous ADP-heptose*, we* observed *considerably weaker* activation of the classical or alternative NF-κB pathways (Fig 1E).*"*

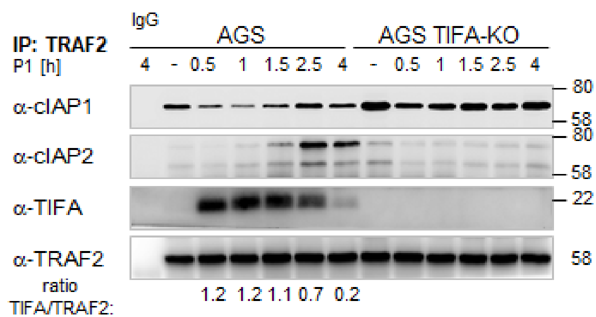
2. Fig2a - the nonspecific band accumulates. Could this band be something relevant, like a different modification?

We are aware of this phenomenon. In experiments using knockdown of p100 by siRNAs, we still observed the unspecific band when we used the antibody specific for the S866/S870 phosphorylation of p100, suggesting that this band has nothing to do with p100 (see the figure below).



3. Figure 3A, 3C, and 3D - The TRAF2 and TIFA interaction kinetics don't agree. In 3A there is a maximum at 30 minutes and almost nothing at 2 hours. In C, the TRAF2/TIFA interaction gives a strong band at 2.5 hours. In D there a maximum intensity band at 1.5 hours and a very strong band at 2.5 hours implying that a 2 hours timepoint would still have a strong band. Since TIFA/TRAF2 interaction kinetics may determine the strength of alternative NfKB pathway activation please comment on these seemingly contradictory kinetics.

We have to distinguish between the immunoblots for co-IP of a His-tagged recombinant protein transfected into the cells by means of protein transfection and co-IPs of endogenous TIFA or TRAF2. We think the co-IP of transfected His-tagged recombinant TIFA cannot be expected to fit 100% with regard to the band intensities with the co-IP of endogenous TIFA. The kinetics of the co-IPs for endogenous TIFA or TRAF2 revealed that the interaction fit well at the early and the later time points. For example, as can be seen from the ratio of TIFA/TRAF2 band intensity quantified using ImageJ (refer to image below) i.e., the intensity of the TIFA-band at 0.5, 1 and 1.5 hours is similar and stronger than at 2.5 hours (Fig. 3D) which agrees with Fig. 3C (the intensity of the TIFA-band at 45 minutes is stronger than at 2.5 hours).



4. 3E is very hard to interpret. The E178A mutant has much less accumulation of NIK in comparison to WT. Why is the if E178 so much weaker if it is important for TRAF6 binding, but not TRAF2? The oligomerization in 3F also looks reduced in comparison to WT. The model described in lines 197-204 may be inadequate.

We have exchanged the immunoblot strip for the α-FLAG-tag antibody and it is clearly more visible now that the expression of the E178A mutant at 2.5 hours is less, compared to the wild-type and T9A mutant (now Fig. 3F). The same is true for the immunoblot of TIFA oligomerization (now Fig. 3G).
We have determined in preceding experiments that using higher plasmid concentrations causes the cells too much stress leading to cell death.

5. Figure 4 - TIFA-tdTomato doesn't look the same in any panel. Especially E. Also, in extended figures 4 and 5 TIFA expression is extremely different between the cells and some cells have undetectable expression in these images. From these images it would be impossible to reproduce the quantitative analysis.

The images in Fig. 4 were of different magnifications. Now, we have modified the images so that they all have the same magnification. For the quantification (Fig. EV4B and Fig. EV5), we used only the ROIs drawn in the images. For better visibility, we have placed the ROI images next to the quantification and labeled them with letters. We used transient expression and therefore some cells do not express TIFA-tdTomato. We have mentioned this aspect throughout the manuscript.

6. The gmhA knockout experiments need further controls. First, experiments using a knockout strain need complementation to address possible polar effects or other genetic interactions. Second, gmhA is a key gene in the LPS biosynthetic pathway. How sick is this strain? An alternative explanation for the observations in figure 1b and 1c is that the knockout strain has growth and virulence defects from perturbations of the outer membrane. In support of this, knockout of gmhA and gmhB seem to have a deficiency in CagA translocation in comparison to an isogenic WT strain. This contributes to a strong reduction in IL-8 secretion despite equivalent levels of CagA expression (2).

We have performed the complementation of the P1 *gmhA*-knockout strain (new Fig. EV1C). The following text was included in the manuscript (Line 129): *"Furthermore, complementation*

of ΔHP0857 with amplified gmhA from the P1 strain rescued the ability to induce both NF-κB pathways (Fig. EV1C)."

1. Fu J, Huang D, Yuan F, Xie N, Li Q, Sun X, Zhou X, Li G, Tong T, Zhang Y. 2018. TRAF-interacting protein with forkhead-associated domain (TIFA) transduces DNA damage-induced activation of NF-κB. J Biol Chem 293:7268-7280.
2. Pfannkuch L, Hurwitz R, Traulsen J, Sigulla J, Poeschke M, Matzner L, Kosma P, Schmid M, Meyer TF. 2019. ADP heptose, a novel pathogen-associated molecular pattern identified in Helicobacter pylori. FASEB J fj.201802555R.

Reviewer #3:

The authors have investigated the role of TIFA in NF-kB signaling in epithelial cells to the bacterial pathogen, Helicobacter pylori. The work presents two major claims. First, it is claimed that H. pylori induction of "TIFasome" formation with the TIFA-interacting partner, TRAF6, leads to the activation of classical NF-kB signaling. The second claim is that TIFA interacts with TRAF2, resulting in proteasomal degradation of cIAP1 and activation of the alternative NF-kB signaling pathway. Although the first claim is not novel within the broad field of TIFA signaling, it does provide new information regarding the H. pylori->TIFA->NF-kB signaling axis. There are currently seven articles (including two reviews) on TIFA and H. pylori in Pubmed, while virtually nothing is known regarding the pathophysiological role of TIFA in H. pylori disease in vivo. The current work should provide impetus for research in this regard. The second claim is of higher novelty and likely to be of broad interest to researchers studying alternative NF-kB signaling.
The data are nicely presented, are of very high quality and consistent with the conclusions drawn. The manuscript is exceptionally well written. The authors have adequately discussed their findings in light of the existing literature, though a couple of important references have been omitted (see comments below). Overall, the manuscript presents new data that are likely to be of interest to those in both the H. pylori field and, more broadly, to those studying NF-kB signaling in response to microbial pathogens. Nevertheless, some additional controls are needed to confirm specificity of the observed phenomena to H. pylori activation of TIFA signaling. Specific points for the authors' consideration are listed below.
Major points:

1) There is no indication regarding the numbers of independent experiments that were performed. This information needs to be supplied for each data set.

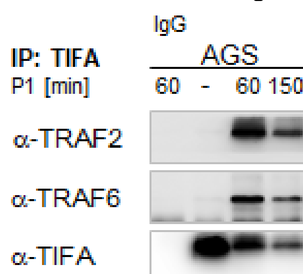
We have included the number of experiments in the figure legends.

2) The authors have confirmed their key findings for AGS cells using multiple cell lines and clones, but have only used one H. pylori strain (P1). As reported previously (Gall et al. doi.org/10.1128/mBio.01168-17), there are significant strain-specific differences in the genes encoding key steps in LPS biosynthesis. These differences may impact ALPK1/TIFA signaling. It is therefore important that the authors test at least an additional pair of H. pylori WT and HP0587 mutant strains, or more than one WT strain, on the AGS, AGS ALPK1-KO and AGS TIFA-KO cell lines (as per Fig. 2A). This will confirm that H. pylori activation of the alternative NF-kB signaling pathway is not a strain-specific phenomenon.

We have performed the infection of AGS, AGS/ALPK1-KO and AGS/TIFA-KO cells as requested with the additional *H. pylori* wild-type strain P12 (new Fig. EV1D) and referenced the data in the manuscript where appropriate.

3) TIFA was shown to interact with TRAF6 and TRAF2 by over-expression using TIFA-KO cells expressing His-tagged recombinant TIFA protein. Although this has been described in other models, there are always concerns regarding over-expression as the sole approach to studying protein interactions. Can endogenous TIFA be pulled down in AGS WT cells stimulated with P1? The anti-TIFA antibody seems of good quality and endogenous TIFA levels seem sufficiently high to be able to do this. Another approach would be to perform the experiment with TIFA-KO cells expressing His-tagged recombinant TIFA, but use HP0587 mutant bacteria.

In Fig. 2C and Fig. 3A, the His-tagged recombinant TIFA protein was transfected, not a plasmid containing the ORF of TIFA. Therefore, we do not really have an overexpression model here in the sense of transcribing and translating TIFA in excess from a plasmid. In Fig. 3B and C, we showed IPs with endogenous TIFA, which fit quite well with the data from the His-IP (Fig. 2C and Fig. 3A). We did not include in the manuscript an IP of endogenous TIFA to show TRAF6 interaction because we deemed it not necessary. For your reference, we show here the endogenous TIFA co-IP detecting TRAF2 and TRAF6.



4) The transfection work with TIFA-dtTomato in AGS-TIFA KO cells (Fig. 4) needs a control. It is not entirely clear, but seems that the observed co-localization of TIFA, TRAF6, TRAF2 etc was in response to H. pylori stimulation (lines 205-208). Nevertheless, TIFA can self-oligomerize (line 85), so it is possible that over-expression may be sufficient to induce TIFAsome formation. The authors need to show TIFAsome formation in the absence of H. pylori stimulation and/or in response to the HP0587 mutant.

We have improved Fig. 4 and show now P1 ΔHP0857 control (new Fig. 4E). The control without P1 has also been included (new Fig. EV4A). We have also included a sentence in the manuscript (Line 243): *"The formation of TIFAsomes was absent in ΔHP0857-infected (Fig. 4E) and uninfected TIFA-KO cells (Fig. EV4A) transiently transfected with TIFA-dtTomato."*

Minor points:

5) TIFA interactions with TRAF2 and TRAF6 have been reported previously (Kanamori et al. BBRC 290, 1108; Takatsuna et al. doi: 10.1074/jbc.M300720200), so it is not possible to claim "...TRAF6 and TRAF2 as NOVEL binding partners of TIFA.." (lines 37-38).

We have omitted the word 'novel'.

6) The Ohmae paper (line 55) described H. pylori activation of the alternative pathway in B lymphocytes and not epithelial cells. It is important to stress this detail in the text. Also, in contrast to the current work, Ohmae reported that H. pylori was unable to induce the alternative pathway in AGS cells. This difference should be discussed.

We have removed the Ohmae paper from the reference list because, as the reviewer has pointed out, they used B-lymphocytes where the NF-κB signaling is different from epithelial cells. That Ohmae *et al.* did not detect the activation of the alternative NF-κB in AGS is probably due to the differences in the signal intensity for p100 processing in B-lymphocytes and AGS (epithelial cells) cells on the same immunoblot. In contrast, they acknowledged that they found *in vivo* some gastric epithelial cells staining for processed p100 (Fig. 7A).

7) The authors need to cite the work of Hirata (DOI: 10.4049/jimmunol.176.6.3796). Those authors first reported the roles of TRAF6 and TAK1 in H. pylori induction of NF-kB signaling in epithelial cells. They also described that this signaling was MyD88-dependent. Do the authors have any data regarding the role of MyD88 in the TIFA-dependent responses observed here?

We have included the citation. No, we did not look for an involvement of MyD88 in our study.

8) The work of Gall et al (mentioned above) should be cited.

We have included the citation.

9) Does TIFA lacking an FHA domain, needed for interactions with TRAF2/TRAF6, have any effect on NF-kB signaling?

The interaction between TRAF6 and TIFA is solely mediated via the E178 of TIFA (Takatsuna *et al*, 2003). The interaction between TRAF2 and TIFA seems to depend on the C-terminal half of TRAF2 and not on the FHA domain of TIFA (Kanamori *et al*, 2002). According to Weng *et al.* (Weng *et al*, 2015), TIFA exists as an intrinsic dimer via homotypic FHA domain interaction, even in the absence of T9 phosphorylation. Upon phosphorylation of T9 in one TIFA dimer, it interacts with the FHA domain of a neighboring dimer leading to oligomerization. Therefore, it can be expected that TIFA lacking the FHA domain is not able to form dimers, does not oligomerize and finally does not induce NF-κB.

10) The HP0858 gene codes for the enzymatic step after HP0587 in the LPS biosynthesis pathway and is the step responsible for the production of the TIFA substrate, HBP. Does the HP0585 mutant induce the same responses in AGS cells as the HP0587 mutant?

We have used the strain harboring the mutant of the apical enzyme (HP0857, *gmhA*) in the cascade for the LPS biosynthesis. It is expected that all enzyme mutants upstream of the ADP-heptose induce the same response since they all are unable to synthesize ADP-heptose.

11) It is unclear why the amounts of the TIFA in this pulldown (Fig. 2E, line 162) are much lower when compared with the other blots.

We observed consistently that the co-IP using the TRAF6 antibody always showed a weaker signal for TIFA as compared to the TRAF2 co-IP, even in the hands of different experimentators.

Gaudet RG, Sintsova A, Buckwalter CM, Leung N, Cochrane A, Li J, Cox AD, Moffat J, Gray-Owen SD (2015) Cytosolic detection of the bacterial metabolite HBP activates TIFA-dependent innate immunity. *Science* 348: 1251-1255

Kanamori M, Suzuki H, Saito R, Muramatsu M, Hayashizaki Y (2002) T2BP, a Novel TRAF2 Binding Protein, Can Activate NF-κB and AP-1 without TNF Stimulation. *Biochem Biophys Res Commun* 290: 1108-1113

Merga YJ, O'Hara A, Burkitt MD, Duckworth CA, Probert CS, Campbell BJ, Pritchard DM (2016) Importance of the alternative NF-κB activation pathway in inflammation-associated gastrointestinal carcinogenesis. *Am J Physiol Gastrointest Liver Physiol* 310: G1081-1090

Pfannkuch L, Hurwitz R, Trauisen J, Sigulla J, Poeschke M, Matzner L, Kosma P, Schmid M, Meyer TF (2019) ADP heptose, a novel pathogen-associated molecular pattern identified in Helicobacter pylori. *The FASEB Journal* 33: 9087-9099

Takatsuna H, Kato H, Gohda J, Akiyama T, Moriya A, Okamoto Y, Yamagata Y, Otsuka M, Umezawa K, Semba K *et al* (2003) Identification of TIFA as an Adapter Protein That Links Tumor Necrosis Factor Receptor-associated Factor 6 (TRAF6) to Interleukin-1 (IL-1) Receptor-associated Kinase-1 (IRAK-1) in IL-1 Receptor Signaling*. *J Biol Chem* 278: 12144-12150

Weng J-H, Hsieh Y-C, Huang C-CF, Wei T-YW, Lim L-H, Chen Y-H, Ho M-R, Wang I, Huang K-F, Chen C-J *et al* (2015) Uncovering the Mechanism of Forkhead-Associated Domain-Mediated TIFA Oligomerization That Plays a Central Role in Immune Responses. *Biochemistry* 54: 6219-6229

Zhou P, She Y, Dong N, Li P, He H, Borio A, Wu Q, Lu S, Ding X, Cao Y *et al* (2018) Alpha-kinase 1 is a cytosolic innate immune receptor for bacterial ADP-heptose. *Nature* 561: 122-126

Zimmermann S, Pfannkuch L, Al-Zeer MA, Bartfeld S, Koch M, Liu J, Rechner C, Soerensen M, Sokolova O, Zamyatina A *et al* (2017) ALPK1- and TIFA-Dependent Innate Immune Response Triggered by the Helicobacter pylori Type IV Secretion System. *Cell Rep* 20: 2384-2395

1st Revision - Editorial Decision 29th Jun 2021

Dear Prof. Naumann,

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, the referees now support the publication of your study in EMBO reports. Referees #3 has some further points and suggestions to improve the manuscript, we ask you to address in a final revised version. Please also provide a point-by-point response regarding these remaining referee concerns.

Moreover, I have these editorial requests I also ask you to address:

- I would suggest a more active title:
TIFA has dual functions in Helicobacter pylori-induced classical and alternative NF-kB pathways

- Please provide the abstract written throughout in present tense.

- Could you use bigger fonts for the labelling of the x- and y-axes of the diagrams shown in figures EV4 and EV5? Presently, the labelling is too small and will hardly be visible in the final online figures.

- Thank you for submitting the source data for the main figures. For the final version, please also submit the Western blot source data for the EV figures (i.e. for all the figures shown in the paper). Please submit one pdf file per figure (main and EV figures).

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with a few changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see any modifications done.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript (35 words).
- three to four bullet points highlighting the key findings of your study.
- a schematic summary figure (synopsis image) in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels that can be used as a visual synopsis on our website.

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

Kind regards,

Achim Breiling
Editor
EMBO Reports

Referee #1:

All concerns have been appropriately addressed, I support publication of this very interesting

manuscript.

Referee #2:

The revision nicely addresses the prior concerns with new experiments and text clarifications.

Referee #3:

The authors have satisfactorily addressed most of the reviewers' comments and questions. They have also included some additional experimental data which has further strengthened the manuscript.
Nevertheless, the authors did not address the issues relating to the viability of the H. pylori gmhA/HP0857 mutant (Reviewer #2, point 6). It has been noted previously that the gmhA mutation has a significant impact on bacterial growth and is even lethal in some H. pylori strains (Gall et al. mBio 8, e01168-17; Stein et al. PLoS Pathogens 13, e1006514; Yu et al. BBRC 477, 794). The authors provided data for a complemented gmhA/HP0587 strain, but did not address this point. They also did not address the Reviewer's comment regarding the fact that the gmhA/HP0857 mutant translocates less CagA than wild type bacteria and that this may also have an impact on IL-8 responses in cells. The gmhA/HP0857 mutant also adheres less to AGS cells.

Related to these points, I also raised the issue of whether an hldE/HP0858 mutant induced the same TIFA-dependent phenotype in cells as the gmhA/HP0857 mutant (point #10) to which the authors responded that "It is expected that all enzyme mutants upstream of the [sic] ADP-Heptose induce the same response....." This seems a reasonable enough assumption, however, it would also be more logical to study a mutant that cannot undergo the key step involved in ADP-Heptose synthesis rather than the one preceding that step.

The above points should be briefly discussed/acknowledged in the manuscript.
For clarity, I also think it would be useful to mention in the main text that His-tagged recombinant TIFA protein was transfected into cells and not DNA (point #3). This is mentioned in the respective figure legends, but not in the text. By definition, "transfection" refers to the introduction of nucleic acids and not proteins into eukaryotic cells.

Point-by-point response to the editorial requests and the reviewer comments

Editorial requests
1 I would suggest a more active title:
TIFA has dual functions in Helicobacter pylori-induced classical and alternative NF-kB pathways

We changed the title as requested.

2 Please provide the abstract written throughout in present tense.

The abstract is now written in present tense.

3 Could you use bigger fonts for the labelling of the x- and y-axes of the diagrams shown in figures EV4 and EV5? Presently, the labelling is too small and will hardly be visible in the final online figures.

We included now in the revised manuscript bigger fonts for the labelling of the x- and y-axes of the diagrams shown in figures EV4 and EV5.

4 Thank you for submitting the source data for the main figures. For the final version, please also submit the Western blot source data for the EV figures (i.e. for all the figures shown in the paper). Please submit one pdf file per figure (main and EV figures).

We provided the Western blot source data for the EV figures and submitted one pdf file per figure (main and EV figures).

5 Finally, please find attached a word file of the manuscript text (provided by our publisher) with a few changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see any modifications done.

We included the requested changes by the publisher in the revised manuscript. The changes are indicated in yellow.

6 In addition, I would need from you:
- a short, two-sentence summary of the manuscript (35 words).
- three to four bullet points highlighting the key findings of your study.
- a schematic summary figure (synopsis image) in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels that can be used as a visual synopsis on our website.

We provided all requests together with the revised manuscript.

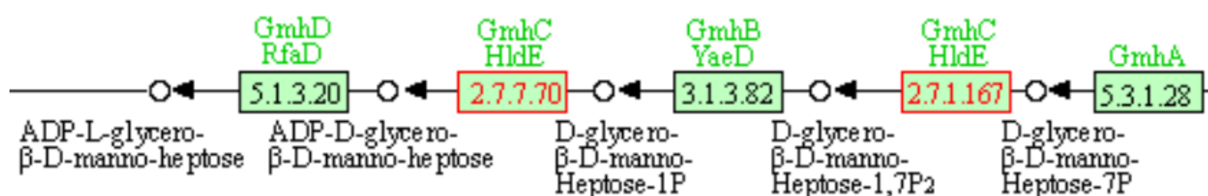
Reviewer #3
1 Nevertheless, the authors did not address the issues relating to the viability of the H. pylori gmhA/HP0857 mutant (Reviewer #2, point 6). It has been noted previously that the gmhA mutation has a significant impact on bacterial growth and is even lethal in some H. pylori strains (Gall et al. mBio 8, e01168-17; Stein et al. PLoS Pathogens 13, e1006514; Yu et al. BBRC 477, 794). The authors provided data for a complemented gmhA/HP0587 strain, but did not address this point. They also did not address the Reviewer's comment regarding the fact that the gmhA/HP0857 mutant translocates less CagA than wild type bacteria and that this may also have an impact on IL-8 responses in cells. The gmhA/HP0857 mutant also adheres less to AGS cells.

We have used the cagA- and T4SS-positive strain P1 of *H. pylori* for most of our experiments. This strain was not used in any of the above-mentioned publications. We did not experience any growth- or adherence defect. In figure EV1C, the intensities of the flagellin bands of the wild-type, the HP0857 mutant and the complemented mutant were similar, which is an indication that adherence is not compromised. Further, based on our experience and published data, the absence of CagA has no impact on NF-κB activation and IL-8 release (e.g. Schweitzer *et al*, 2010).
We now acknowledged the observations by other groups in the main text of the revised manuscript as follows (lines 125-128): *“Of note, an impact on viability or adherence as*

described for HP0857 mutants in other H. pylori strains (Gall et al, 2017; Stein et al, 2017; Yu et al, 2016) were not observed for the P1 strain used here."

2 Related to these points, I also raised the issue of whether an hldE/HP0858 mutant induced the same TIFA-dependent phenotype in cells as the gmhA/HP0857 mutant (point #10) to which the authors responded that "It is expected that all enzyme mutants upstream of the [sic] ADP-Heptose induce the same response....." This seems a reasonable enough assumption, however, it would also be more logical to study a mutant that cannot undergo the key step involved in ADP-Heptose synthesis rather than the one preceding that step.

The hldE/HP0858 enzyme of *H. pylori* is bifunctional, meaning it is involved in two steps of the biosynthesis of ADP-heptose, although with different activities. Therefore, we have decided to mutate *gmhA* to silence the whole biosynthesis of ADP-heptose.



We have provided our reason for using the ΔHP0857 mutant by revising the sentence in the main text as follows (lines 122-123): "To investigate whether ALPK1 and TIFA are involved in the activation of the alternative NF-κB pathway, we used the *H. pylori* mutant strain (ΔHP0857), *where gmhA was mutated leading to the termination of the whole biosynthesis pathway of ADP-heptose."*

3 The above points should be briefly discussed/acknowledged in the manuscript.
For clarity, I also think it would be useful to mention in the main text that His-tagged recombinant TIFA protein was transfected into cells and not DNA (point #3). This is mentioned in the respective figure legends, but not in the text. By definition, "transfection" refers to the introduction of nucleic acids and not proteins into eukaryotic cells.

As requested we have now acknowledged the raised points in the revised manuscript.

Presumably the reviewer has overseen that we had already mentioned in our first submitted manuscript (line 152) that His-tagged recombinant TIFA protein was transfected into cells.

Gall A, Gaudet RG, Gray-Owen SD, Salama NR (2017) TIFA Signaling in Gastric Epithelial Cells Initiates the cag Type 4 Secretion System-Dependent Innate Immune Response to Helicobacter pylori Infection. mBio 8: e01168-01117

Schweitzer K, Sokolova O, Bozko PM, Naumann M (2010) Helicobacter pylori induces NF-κB independent of CagA. EMBO Rep 11: 10-11

Stein SC, Faber E, Bats SH, Murillo T, Speidel Y, Coombs N, Josenhans C (2017) Helicobacter pylori modulates host cell responses by CagT4SS-dependent translocation of an intermediate metabolite of LPS inner core heptose biosynthesis. PLoS Pathog 13: e1006514

Yu C-K, Wang C-J, Chew Y, Wang P-C, Yin H-S, Kao M-C (2016) Functional characterization of Helicobacter pylori 26695 sedoheptulose 7-phosphate isomerase encoded by hp0857 and its association with lipopolysaccharide biosynthesis and adhesion. Biochem Biophys Res Commun 477: 794-800

2nd Revision - Editorial Decision 9th Jul 2021

Prof. Michael Naumann
Otto von Guericke University
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Magdeburg
Germany

Dear Prof. Naumann,

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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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 χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

B- Statistics and general methods

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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	N/A
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Experiments were chosen based on quality control including the check if infection was effective by an appropriate activation of signal transmission (detection of protein modifications, protein translocation etc.). Further quality control included for the comparison of different samples the measurement of protein concentrations in cell lysates.
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.	Subjective bias was avoided by collecting corroborating data sets from alternative experimental approaches and by performing the experiments by different experimenators.
For animal studies, include a statement about randomization even if no randomization was used.	N/A
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.	N/A
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
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.	N/A

Is there an estimate of variation within each group of data?	N/A
Is the variance similar between the groups that are being statistically compared?	N/A

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).	All antibodies were received commercially from a variety of suppliers and listed in the Materials and Methods section of the manuscript. The antibodies are approved by the suppliers and in addition all antibodies were approved for use in our experiments.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The AGS and HeLa cell lines were received from the German Collection of Microorganisms and Cell Cultures GmbH and are routinely tested for mycoplasma contamination in the lab.

* 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.	N/A
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
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.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
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.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
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.	N/A
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.	N/A

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	N/A
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).	Relevant datasets have been provided in "Expanded View" and "Source Data"
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).	N/A
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.	N/A

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.	N/A