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Infection-driven activation of transglutaminase 2 boosts glucose uptake and hexosamine biosynthesis in epithelial cells

Benoit Maffei, Marc Laverriere, Yongzheng WU, Sébastien Triboulet, Stéphanie Perrinet, Magalie Duchateau, Mariette Matondo, Robert Hollis, Charlie Gourley, Jan Rupp, Jeffrey Keillor, and Agathe Subtil.

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

1st Editorial Decision 1st May 2019

Thank you for submitting your manuscript on a role for transglutaminase 2 (TG2) in regulating the development of Chlamydia trachomatis to The EMBO Journal. Your study has been sent to three referees for evaluation, and their reports are enclosed below for your information.

As you can see, while all referees consider the findings novel and potentially interesting, they also raise several points that need to be addressed before they can support publication in The EMBO Journal. In particular, referee #1 asks you to clarify how TG2 regulates the transcription of glucose transporters in infected cells and the underlying transcriptional mechanisms. Referee #2 requests you to show how bacteria increase TG2 expression and to test the specificity of CP4d inhibitor. Referee #3 asks you to compare the effects of TG2 inhibition and knockdown on bacterial size/cell division.

Given the overall interest of your study, I would like to invite you to submit a revised version of the manuscript according to the referees' requests.

REFEREE REPORTS

Referee #1:

The ms by Maffei et al. entitled "Infection-driven activation of transglutaminase 2 boosts glucose uptake and hexosamine biosynthesis "shows that the genetic or pharmacological inhibition of TG2 activity impairs the bacterial development of Chlamydia trachomatis. Furthermore, the authors

show that TG2 activation drives one specific glucose-dependent pathway in the host. In fact, they identified the glucosamine:fructose-6-phosphate amidotransferase (GFPT) as a substrates of TG2. GFPT modification by TG2 increases its enzymatic activity resulting in higher levels of UDP-N-acetylglucosamine biosynthesis. In general, this a novel and interesting story that confirms and extends the pathgenetic involvement of TG2 in the modulation of the host's immunity against intracellular bacteria. The paper is well written and the results support the conclusions. However, in order to improve the impact of the present study the authors should answer to the following points:
1. The authors show that, in the presence of the TG2 inhibitor CP4d or TG2 KO cells, the transcription of the glucose transporter genes was no longer induced by the infection, thus indicating that TG2 is necessary for the control of GLUT-1 and GLUT-3 transcription. In line with this assumption they should demonstrate how TG2 regulates the transcription of these glucose transporters. The study proposes that the TG2-dependent constitutive NF-kB activation by promoting the expression of the HIF in turn might enhance GLUT-1 transcription. It well known that TG2 can regulate gene expression by modifying many key transcriptional factors such as SP1 and HSF1 and recently TFIID via the serotonylation of H3K4me3. Thus, in order to demonstrate the role of TG2 in the Chlamidia infection it is important to explain which transcriptional mechanisms is involved in the induction of the expression of these genes.
2. The authors identified GFPT as a potential TG2 substrate and they also report a three-fold increase in its activity in cells treated with ionomycin which, by increasing the intracellular calcium concentration, should activate TG2 transamidating activity. However, the authors should characterize the type of post-translational modification catalyzed by TG2 (tetramerization, amine incorporation, deamination, serotonylation ect) which is responsible for the GFPT activation.
3. Although the authors emplyed MEFs and epithelial cells in the reported experiments, most of the work was carried out using a highly trasformed cell line: HeLa cells. The authors should explain the rationale for selecting such cell line.
4. Considering that the TG2 inhibitor CP4d cannot be used as therapeutic agent, it would be useful to test cysteamine that is the only TG2 inhibitor approved by both FDA and EMA.

Referee #2:

Comments to Authors:

The manuscript "Infection-driven activation of transglutaminase 2 boosts glucose uptake and hexosamine biosynthesis" by Maffei and coworkers describes a relationship between infection of cells by chlamydia, TG2 expression and activation, and cellular metabolic alterations. The authors describe how their findings show that TG2 controls glucose-mediated metabolic pathways in mammalian cells and highlight an unanticipated connection between the transamidase activity of TG2 and O-GlcNAcylation, which is disrupted upon bacterial infection because the bacteria hijack this metabolic pathway to assist their cell division. Overall, this makes for an interesting story. TG2 is widely expressed in several different disease states, and metabolic reprogramming is a hallmark of various types of aberrant biology and disease. While the link between TG2 and glucose uptake has been previously described, the connection to the hexosamine pathway appears to be novel. However, as listed below, there are some issues that the authors need to consider and are encouraged to respond to prior to publication. For example, it is possible that ionomycin treatment causes GFPT activity to increase via TG2 involvement, but flooding a cell with calcium might have multiple effects in addition to activating TG2. The amounts of CP4d being used are also very high, leading to questions about the specificity of its action and the conclusions drawn from those results in which a complementary knockdown of TG2 was not performed. It also would be of interest to know how bacteria cause TG2 expression to increase, and how TG2 exerts its various effects on GLUT-1, GLUT-3, and GFPT.

Still despite these issues, the manuscript presents findings that should be of interest to a wide audience, as it touches upon topics of immediate relevance to researchers examining bacterial infection, metabolic alterations, and several important enzymes. Thus on balance, assuming that the authors can address the points raised below, this study should be appropriate for The EMBO Journal.

Major issues:

1) Fig. 1A shows that the bands at 125 and 75 kDa are not due to BP, yet it also shows that 40 μM CP4d is able to inhibit the development of the 75 kDa band in uninfected cells. Moreover, Fig. 1B shows that the 75 kDa band only shows up when BP is present. This appears to be confusing and needs clarification.

2) Western blots should show several bandwidths above and below the chosen band. However, for many figures such as TG2 in Fig. 1D, the bands are cut off by the cropping.

3) What is known about the specificity of CP4d for TG2? Ideally, one would like to see that any effects observed with CP4d, especially given the relatively high levels used, are matched by TG2 knock-downs.

4) Re: Fig. 2A/B, is there any way to measure the survival of the bacteria in the presence of CP4d, while eliminating host cells from the equation? The bacteria may simply be dying due to the drug, thus decreasing their infectivity.

5) What are p-values measuring in Fig. 3C? There are not two obvious cohorts - what statistical significance is being examined?

6) Fig. 4A needs a beads control (pulldown with beads and protein, but no antibody) for both + and - drug conditions.

7) Data in 4D would be more compelling if the data were less blurry and not so tightly cropped.

8) Why are there no error bars for the control experiment in Fig. 4D? Similarly, BP inclusion looks reduced in Q58N as well - what was the stat. significance of the difference there?

9) Fig. 4D also begs the question: which glutamines are responsible for the other ~60-70% of TG2 modification?

10) Fig. 5C requires + and - ionomycin in the TG2 knockdown. It also requires a second siRNA, and a matching blot to show the level of TG2 reduction.

11) Do authors have access to a better GFPT antibody? Figs. 6A and 6B look more like smears than bands, making it difficult to accurately assess the level of GFPT expression.

12) As the authors point out in their "Discussion', one would really like to know the amine donor for GFPT. I can give the authors the benefit of the doubt regarding this being the objective of future studies, but do they have any intriguing candidates that they might at least mention in their 'Discussion'?

13) Ideally, it would strengthen the authors' arguments if they were able to show in a reconstituted model system using recombinant proteins that TG2-catalyzed crosslinking of GFPT enhances its activity.

14) How do the authors imagine that bacteria increase TG2 expression? While it might be assumed that this is similar, or identical, to other mechanisms by which inflammation causes TG2 expression to increase, one would have like to have seen that addressed for these studies.

Minor issues:

1) The journal policy is to cite primary data, not reviews. It might be best to replace many of these citations with the articles in which the discussed findings originally appeared.

2) Similarly, all p-values described as * or the like should be replaced with the actual p-value determined.

3) Catalog numbers for secondary antibodies should be included.

4) The IACUC protocol number, or other authorizing body for mouse experiments/animal welfare, should be provided in the "Infection in mice" portion of the methods. The only information provided is for Dr. Papista, who provided the mice. It appears that there is no information provided for the actual experiments performed.

Referee #3:

This work addresses the activation and function of human transglutaminase-2 (TG2) during infection by Chlamydia. The authors show that TG2 activity increases in Chlamydia-infected cells and that this activation is important for the bacterial infectious cycle. The experiments presented support a model in which this activation of TG2 promotes Chlamydia infection by: (i) increasing the levels of the host glucose transporters GLUT1 and GLUT3; and thus increasing the availability of glucose to the bacteria): (ii) increasing the activity of host glucosamine:fructose-6-phosphate (GFPT), which leads to higher levels of UDP-N-acetylglucosamine that are used during bacterial cell division for LPS and peptidoglycan biosynthesis.

In general, this is an exciting work of high quality with impact not only in the Chlamydia field but in the more general context of the interaction between pathogens and host metabolism. It also suggests additional unexplored functions of TG2 in uninfected cells.

Major issue:

1. Based on the model in Figure 6F, it is expected that pharmacological or siRNA inhibition of TG2 would impact on bacterial size/cell division similarly to silencing of GFPT. This would provide one additional and important evidence to support the proposed model. Might not be straightforward, because not having the higher expression of GLUT1 and GLUT3 could also impact on bacterial size/cell division but this could be discussed depending on the outcome of the experiments.

Minor issues:

2. The title should be modified to better reflect the nature of the work and its conclusions. While the work is of broad impact, what is studied here is mostly in the context of Chlamydia infection and how TG2 activation is explored by the bacteria.

3. Some of the Western blots could be of better quality and should be remade: anti-GFPT in Figure 6A and 6B, and anti-actin in Figure 6C.

4. I suppose the blot in Figure 1D was repeated a few times. Using the available data, a quantification of the anti-TG2 signal vs anti-actin could further strengthen the conclusions.

5. Does inhibition of TG2 also affect C. muridarum infection of HeLa cells? Given that mice infections were done with C. muridarum it would be nice to have this additional information.

6. The authors use both pharmacological inhibition of TG2 and fibroblasts from tgm2 knockout mice to show the importance of the enzyme during C. trachomatis infection. One additional control would be to test this in the tgm2-silenced cells they use also in the work. I think the evidence already presented is strong, but it is a bit weird that pharmacological inhibition was used instead of tgm2 siRNA silencing to test this.

Referee #1:

We thank reviewer #1 for his/her time and careful evaluation of our findings. Answers to his/her questions are in blue below.

The ms by Maffei et al. entitled "Infection-driven activation of transglutaminase 2 boosts glucose uptake and hexosamine biosynthesis " shows that the genetic or pharmacological inhibition of TG2 activity impairs the bacterial development of Chlamydia trachomatis. Furthermore, the authors show that TG2 activation drives one specific glucose-dependent pathway in the host. In fact, they identified the glucosamine:fructose-6-phosphate amidotransferase (GFPT) as a substrates of TG2. GFPT modification by TG2 increases its enzymatic activity resulting in higher levels of UDP-N-acetylglucosamine biosynthesis. In general, this a novel and interesting story that confirms and extends the pathgenetic involvement of TG2 in the modulation of the host's immunity against intracellular bacteria. The paper is well written and the results support the conclusions. However, in order to improve the impact of the present study the authors should answer to the following points:

1. The authors show that, in the presence of the TG2 inhibitor CP4d or TG2 KO cells, the transcription of the glucose transporter genes was no longer induced by the infection, thus indicating that TG2 is necessary for the control of GLUT-1 and GLUT-3 transcription. In line with this assumption they should demonstrate how TG2 regulates the transcription of these glucose transporters. The study proposes that the TG2-dependent constitutive NF-kB activation by promoting the expression of the HIF in turn might enhance GLUT-1 transcription. It well known that TG2 can regulate gene expression by modifying many key transcriptional factors such as SP1 and HSF1 and recently TFIID via the serotonylation of H3K4me3. Thus, in order to demonstrate the role of TG2 in the Chlamidia infection it is important to explain which transcriptional mechanisms is involved in the induction of the expression of these genes.

Indeed, TG2 acts on several transcriptional networks. Our hypothesis that HIF-1α might be implicated was based on previous demonstration of its upregulation in *Chlamydia* infected cells (Sharma 2011), and by the detailed work by the Mehta group, showing that expression of TG2 induced that of HIF-1α under normoxic conditions, and that this transcription factor was required for the increase in glucose uptake in mammary epithelial cells (Kumar 2014). We thus tested this hypothesis, and observed that the increase in GLUT-1 and GLUT-3 transcription in infection was lost in cells in which HIF-1α expression had been silenced. These data support the hypothesis that TG2 induction of GLUT-1 and GLUT-3 transcription is mediated by an increase in HIF-1α expression and are now displayed in Fig. 3C.

2. The authors identified GFPT as a potential TG2 substrate and they also report a three-fold increase in its activity in cells treated with ionomycin which, by increasing the intracellular calcium concentration, should activate TG2 transamidating activity. However, the authors should characterize the type of post-translational modification catalyzed by TG2 (tetramerization, amine incorporation, deamination, serotonylation ect) which is responsible for the GFPT activation.

The absence of shift in the migration profile of GFPT suggests that the amine donor is either a small protein, or a small amine, or that deamidation occurs. Looking for the unknown by mass spectrometry is very challenging. For the revision of this manuscript we have immunoprecipitated GFPT after ionomycin treatment, in an attempt to enrich in modified peptides. Although we obtained an excellent coverage of GFPY by this approach, we did not detect histaminylation nor serotonylation on any glutamine residue. Deamidation was occasionally seen, on several glutamine residues, including Q328, raising questions as to the relevance of this observation. These observations are now included in the discussion of the revised version. We think that optimization of the procedure is still required to definitively identify the nature of the modification on GFPT, and this important question will be addressed in future studies.

3. Although the authors emplyed MEFs and epithelial cells in the reported experiments, most of the work was carried out using a highly trasformed cell line: HeLa cells. The authors should explain the rationale for selecting such cell line.

HeLa cells derive from cervical epithelial cells, which is a well documented niche for *C. trachomatis* multiplication. For this reason, and for their easy manipulation, HeLa constitute the main cell line used in *Chlamydia* research. However, it is necessary to verify the finding made in this highly mutagenized background in primary cells, especially regarding metabolic regulation. This is why all key experiments were also done in primary cells, as well as using MEFs.

4. Considering that the TG2 inhibitor CP4d cannot be used as therapeutic agent, it would be useful to test cysteamine that is the only TG2 inhibitor approved by both FDA and EMA.

We have tested cysteamine and also observed a decrease in the production of infectious bacteria in a dose dependent manner. These data are now displayed in Fig. S2A

Referee #2:

We thank reviewer #2 for his/her time and careful evaluation of our findings. Answers to his/her questions are in blue below.

Comments to Authors:

The manuscript "Infection-driven activation of transglutaminase 2 boosts glucose uptake and hexosamine biosynthesis" by Maffei and coworkers describes a relationship between infection of cells by chlamydia, TG2 expression and activation, and cellular metabolic alterations. The authors

describe how their findings show that TG2 controls glucose-mediated metabolic pathways in mammalian cells and highlight an unanticipated connection between the transamidase activity of TG2 and O-GlcNAcylation, which is disrupted upon bacterial infection because the bacteria hijack this metabolic pathway to assist their cell division. Overall, this makes for an interesting story. TG2 is widely expressed in several different disease states, and metabolic reprogramming is a hallmark of various types of aberrant biology and disease. While the link between TG2 and glucose uptake has been previously described, the connection to the hexosamine pathway appears to be novel. However, as listed below, there are some issues that the authors need to consider and are encouraged to respond to prior to publication. For example, it is possible that ionomycin treatment causes GFPT activity to increase via TG2 involvement, but flooding a cell with calcium might have multiple effects in addition to activating TG2. The amounts of CP4d being used are also very high, leading to questions about the specificity of its action and the conclusions drawn from those results in which a complementary knockdown of TG2 was not performed. It also would be of interest to know how bacteria cause TG2 expression to increase, and how TG2 exerts its various effects on GLUT-1, GLUT-3, and GFPT.

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Major issues:

1) Fig. 1A shows that the bands at 125 and 75 kDa are not due to BP, yet it also shows that 40 ⌈M CP4d is able to inhibit the development of the 75 kDa band in uninfected cells. Moreover, Fig. 1B shows that the 75 kDa band only shows up when BP is present. This appears to be confusing and needs clarification.
The 120 and 75 kDa bands have been identified in rat liver as being biotinylated pyruvate carboxylase and methylcrotonyl-CoA carboxylase respectively (Haneji, T., and Koide, S. S. (1989) Transblot identification of biotin-containing proteins in rat liver. Anal. Biochem. 177, 57-61, doi:10.1016/0003-2697(89)90013-4). We consistently see these two bands in the absence of BP, but with varying intensity between experiments. Fig. 1B indicates that there might be an additional band also migrating at 75 kDa that appears upon BP addition. This interpretation also fits with the CP4d sensitive increase in intensity in this band observed in Fig. 1A. We have added the reference to the publication reporting the identification of the two biotinylated proteins in the legend of the figure for clarification.

2) Western blots should show several bandwidths above and below the chosen band. However, for many figures such as TG2 in Fig. 1D, the bands are

cut off by the cropping.
We have changed the images to less-cropped version in Fig. 1D, Fig. 4D, Fig. 6A and 6B.

3) What is known about the specificity of CP4d for TG2? Ideally, one would like to see that any effects observed with CP4d, especially given the relatively high levels used, are matched by TG2 knock-downs.
We verified that silencing TG2 using two different siRNAs reproduced the effects observed with CP4d, both on *C. trachomatis* and *C. muridarum*. These data are now displayed in Fig. 2D, Fig. S3, Fig. 3B and Fig. 5C. Fig. 6C also now displays data obtained with TG2 silencing.

4) Re: Fig. 2A/B, is there any way to measure the survival of the bacteria in the presence of CP4d, while eliminating host cells from the equation? The bacteria may simply be dying due to the drug, thus decreasing their infectivity.
Because *C. trachomatis* are obligate intracellular bacteria, it is not possible to measure bacterial fitness in the absence of host cell. The experiments using TG2 KO MEF cells (Fig. S2), as well as the new data using silencing of TG2 instead of the drug, strongly support the hypothesis that CP4d does not restrict bacterial growth by directly killing the bacteria, but through its action on TG2 activity.

5) What are p-values measuring in Fig. 3C? There are not two obvious cohorts - what statistical significance is being examined?
These values are from Spearman's rank concordance correlation coefficient, demonstrating the strength (ρ) and significance of the association between GLUT1 and TG2 mRNA expression ($\rho=0.21$, $P<0.001$) and GLUT3 and TG2 mRNA expression ($\rho=0.50$, $P<0.001$) within the high grade serous ovarian cancer cohort. Spearman's rank correlation was chosen over Pearson's correlation following demonstration of non-normal expression distribution for TG2, GLUT1 and GLUT3 (Shapiro-wilk normality test, $P<0.05$ for all). This is now specified in the Methods section.

6) Fig. 4A needs a beads control (pulldown with beads and protein, but no antibody) for both + and - drug conditions.
Fig. 4A is not an immunoprecipitation with an antibody, but a pull-down of biotinylated proteins with streptavidin-coupled beads, after incorporation of biotin pentylamine in TG2 substrates. The pull-downed fraction was run on a gel, transferred on a membrane, and probed with anti-GFPT antibodies. The presence of a signal in the pulled-down fraction, and, most importantly, the observation that less GFPT was present in the biotinylated fraction in the lysates of cells incubated with CP4d, supported the proteomics based hypothesis that GFPT was a TG2 substrate. The follow-up experiments, displayed in Fig. 4 and 5, further confirmed this finding.

7) Data in 4D would be more compelling if the data were less blurry and not so tightly cropped.
The images we show use the best resolution given by our camera, so this aspect cannot be improved. However, we now show less cropped images. Also, we realized that we had made a mistake when designating the third glutamine that we highlight in this figure: we had studied Q555 and not Q546. This has been corrected in Fig. 4 and in the text.

8) Why are there no error bars for the control experiment in Fig. 4D? Similarly, BP inclusion looks reduced in Q58N as well - what was the stat. significance of the difference there?

For this panel we now show all the p-values, even if only the Q328N mutant displays a p-value <0.05. Ratios of streptavidin to anti-GFPT are expressed relative to the ratio measured with WT protein, which is set to 100. This is the reason why there is no error bar for the data using the WT.

9) Fig. 4D also begs the question: which glutamines are responsible for the other ~60-70% of TG2 modification?

It is likely that promiscuous reactions occur *in vitro*, on glutamine residues that are not relevant in physiological reactions. Any (and likely several) of the 9 other glutamine residues in which BP incorporation was detected by mass spectrometry after the reaction *in vitro* (displayed in blod in Fig. 4C) could account for the remaining BP incorporation.

10) Fig. 5C requires + and - ionomycin in the TG2 knockdown. It also requires a second siRNA, and a matching blot to show the level of TG2 reduction.

The experiment has been repeated following this comment, and is displayed in a revised Fig. 5C. The efficiency of the depletion using siRNA against TG2 is shown in Fig. 1B

11) Do authors have access to a better GFPT antibody? Figs. 6A and 6B look more like smears than bands, making it difficult to accurately assess the level of GFPT expression.

Uncropped blots of better quality are now displayed in Panels 6A and 6B.

12) As the authors point out in their "Discussion', one would really like to know the amine donor for GFPT. I can give the authors the benefit of the doubt regarding this being the objective of future studies, but do they have any intriguing candidates that they might at least mention in their 'Discussion'?

The absence of shift in the migration profile of GFPT suggests that the amine donor is either a small protein, or a small amine, or that deamidation occurs. Looking for the unknown by mass spectrometry is very challenging. For the revision of this manuscript we have immunoprecipitated GFPT after ionomycin treatment, in an attempt to enrich in modified peptides. Although we obtained an excellent coverage of GFPY by this approach, we did not detect histaminylation nor serotonylation on any glutamine residue. Deamidation was occasionally seen on several glutamine residues, including Q328, raising questions as to the relevance of this observation. These observations are included in the discussion of the revised version. We think that optimization of the procedure is still required to definitively identify the nature of the modification on GFPT, and this important question will be addressed in future studies.

13) Ideally, it would strengthen the authors' arguments if they were able to show in a reconstituted model system using recombinant proteins that TG2-catalyzed crosslinking of GFPT enhances its activity.

We agree that it would be ideal, but difficult to realize until we know the nature of the amine donor. In addition, it will probably be difficult to obtain sufficient GFPT modified at the target site to be able to detect a change in activity without having concomitant non-specific cross-linking at neighboring sites, that might negatively affect the enzyme's activity.

14) How do the authors imagine that bacteria increase TG2 expression? While it might be assumed that this is similar, or identical, to other mechanisms by which inflammation causes TG2 expression to increase, one would have like to have seen that addressed for these studies.

We tested the hypothesis that IL-6 might be implicated in the transcriptional up-regulation of TG2 in *Chlamydia* infected cells, since this cytokine is produced by epithelial cells during infection. We verified that TG2 transcription showed a dose-dependent response to the addition of recombinant IL-6 in the culture medium. Furthermore, we observed a reduction of TG2 transcription in infected cells with increasing concentrations of anti-IL-6 receptor antibodies in the culture medium. These data indicate that, in the case of *C. trachomatis* infection, IL-6 plays a key role in mediating the inflammation-induced up-regulation of TG2 expression, and are now displayed in Fig. 1F and 1G.

Minor issues:

1) The journal policy is to cite primary data, not reviews. It might be best to replace many of these citations with the articles in which the discussed findings originally appeared.

We have replaced 3 review articles by the original articles (one occurence of Gundemir 2012 replaced by Folk 1967, Love 2005 replaced by Kreppel 1999, Derré 2015 replaced by Derré 2011). The other review articles recapitulate a lot of work in different models. For the sake of the length of the reference section they cannot be replaced by original papers.

2) Similarly, all p-values described as * or the like should be replaced with the actual p-value determined.

This has been done.

3) Catalog numbers for secondary antibodies should be included.

This has been done.

4) The IACUC protocol number, or other authorizing body for mouse experiments/animal welfare, should be provided in the "Infection in mice" portion of the methods. The only information provided is for Dr. Papista, who provided the mice. It appears that there is no information provided for the actual experiments performed.

The appropriate protocol number has been added in the Methods section.

Referee #3:

This work addresses the activation and function of human transglutaminase-2 (TG2) during infection by Chlamydia. The authors show that TG2 activity increases in Chlamydia-infected cells and that this activation is important for the bacterial infectious cycle. The experiments presented support a model in which this activation of TG2 promotes Chlamydia infection by: (i) increasing the levels of the host glucose transporters GLUT1 and GLUT3; and thus increasing the availability of glucose to the bacteria): (ii) increasing the activity of host glucosamine:fructose-6-phosphate (GFPT), which leads to higher levels of UDP-N-acetylglucosamine that are used during bacterial cell division for LPS and peptidoglycan biosynthesis.

In general, this is an exciting work of high quality with impact not only in the Chlamydia field but in the more general context of the interaction between pathogens and host metabolism. It also suggests additional unexplored functions of TG2 in uninfected cells.

Major issue:

1. Based on the model in Figure 6F, it is expected that pharmacological or siRNA inhibition of TG2 would impact on bacterial size/cell division similarly to silencing of GFPT. This would provide one additional and important evidence to support the proposed model. Might not be straightforward, because not having the higher expression of GLUT1 and GLUT3 could also impact on bacterial size/cell division but this could be discussed depending on the outcome of the experiments.
We performed the suggested experiment and observed that TG2 silencing also resulted in an increase in bacterial size, to a similar extent as what we had seen for GFPT silencing. These data are now displayed in Fig. 6D.

Minor issues:

2. The title should be modified to better reflect the nature of the work and its conclusions. While the work is of broad impact, what is studied here is mostly in the context of Chlamydia infection and how TG2 activation is explored by the bacteria.
If we put more emphasis on the infectious side of our work than we currently do in our title, we might lose the attention of part of the EMBO Journal readership. Because we think that our findings should reach the community interested in metabolism regulation at large, we would really prefer to keep the present title.

3. Some of the Western blots could be of better quality and should be remade: anti-GFPT in Figure 6A and 6B, and anti-actin in Figure 6C.
Panels 6A and 6B were modified. We did not repeat the experiment shown in Fig. 6C because, although we agree that the actin blot is not optimal, we think that the data clearly

demonstrate that GFPT1 is by far the main isoform expressed in HeLa cells, and that our siRNAs work.

4. I suppose the blot in Figure 1D was repeated a few times. Using the available data, a quantification of the anti-TG2 signal vs anti-actin could further strengthen the conclusions.
Quantification was performed and is now displayed in 1D.

5. Does inhibition of TG2 also affect C. muridarum infection of HeLa cells? Given that mice infections were done with C. muridarum it would be nice to have this additional information.
These data are now displayed in Fig. S3. Basically, inhibition of TG2 as the same effect on *C. muridarum* as on *C. trachomatis*.

6. The authors use both pharmacological inhibition of TG2 and fibroblasts from tgm2 knockout mice to show the importance of the enzyme during C. trachomatis infection. One additional control would be to test this in the tgm2-silenced cells they use also in the work. I think the evidence already presented is strong, but it is a bit weird that pharmacological inhibition was used instead of tgm2 siRNA silencing to test this.
Silencing TG2 using two different siRNAs reproduced the effects observed with CP4d, both on *C. trachomatis* and *C. muridarum*. These data are now displayed in Fig. 2B, Fig. S3, Fig. 3B and Fig. 5C.

Reference: EMBOJ-2019-102166 “Infection-driven activation of transglutaminase 2 boosts glucose uptake and hexosamine biosynthesis” by Maffei et al.

Dear Editor,

We were pleased to hear that Reviewer #2 and #3 endorsed our paper for publication, and we thank you for giving us a chance to reply to Reviewer#1’s concerns.

Please find below my response to his/her comments:

The new version of the paper entitled "nfection-driven activation of transglutaminase 2 boosts glucose uptake and hexosamine biosynthesis" by Agathe Subtil et al. is substantially improved upon revision, however the authors did not address the two major points I raised in my comments. I think these points need to be clarified in order to render this manuscript suited for publications in EMBO Journal.

1. The authors have performed an experiment in which, by silencing HIF-1alpha, observed a reduction in GLUT-1 and GLUT-3 transcription. This is not a direct evidence of the fact that this is the pathway regulated by TG2. The authors must demonstrate in which way does TG2 regulate/modify this transcriptional factor during the bacterial infection.

In our revised version, we had shown that the transcription factor HIF-1 was necessary, since silencing HIF-1α abrogated infection-induced up-regulation of GLUT-1 and GLUT-3 in HeLa cells. The increase in HIF-1α level during *Chlamydia trachomatis* infection was already published (1), but whether this was accompanied with an increase at the transcriptional level had not been tested. We now show that *HIF-1α* transcripts are stable in infection, demonstrating that the increase in protein level is not due to a transcriptional up-regulation (new Fig. 3D). This is in contrast to the TG2-driven upregulation of GLUT-1 by TG2 in mammary epithelial cells, in which an increase in *HIF-1α* transcripts is observed (2). The second piece of data described below confirms that, contrary to our expectation, and in spite of the facts that the players are the same (TG2, HIF-1), the molecular details are different in *Chlamydia* infected cells and other contexts studied so far.

Indeed, the main point that we had not addressed in the revision was whether the transamidase activity of TG2 was implicated in the transcriptional up-regulation of glucose transporters. Because we had shown that the transamidase inhibitor CP4d inhibited the transcriptional up-regulation of TG2 itself (Fig. 1E) it was not possible to know whether the transamidase activity was implicated in the downstream step, i.e. transcriptional up-regulation of glucose transporters. To answer this key question we now used TG2 $^{-/-}$ MEFs with constitutive expression of wild type TG2, or of a point-mutant of TG2 that has no transamidase activity (C277S mutant (3)). As expected, infection failed to induce the transcription of

GLUT-1 in TG2 $^{-/-}$ MEFs. Constitutive expression of wild type TG2 restored the induction, confirming the role of TG2 in the transcriptional control of glucose transport in the infectious context. Very importantly, the C277S mutant of TG2 failed to restore the infection-induced up-regulation of glucose transporters, demonstrating that the transamidase activity of TG2 is necessary in this context (New Fig. 3F). This is in contrast with other situations where TG2 was shown to modulate HIF-dependent transcription in a transamidase-independent manner (4-6).

This new piece of data is very important, and we thank Reviewer #1 for having encouraged us to dig further in this direction, as the data demonstrate that we are dealing with an as yet undescribed mechanism of HIF-1 dependent/ transglutaminase-controlled transcription of glucose transporters.

In spite of this important addition, Reviewer #1 might still be reluctant to endorse publication of our report in *The EMBO Journal*, based on the fact that we do not identify the TG2 target(s) that lies between TG2 activity and transcriptional control of *GLUT-1* and *GLUT-3* during infection. If you allow me, I will try to convince him/her below that this would be a request for a whole new project and therefore a different paper.

To put it in a nutshell, the identification of the TG2 substrate that eventually leads to the induction of GLUT-1 and GLUT-3 is a whole research project of its own, and definitively a difficult one. None of the TG2 substrates that were recovered in our proteomic approach appears as a good candidate. In the absence of candidate, there is no robust method to address this question, especially when we are looking for unknown post-translational modification of a TG2 substrate. The transamidation of the substrate might change its activity without affecting its abundance, or even its DNA binding activity (if the substrate is a transcription factor), making its identification extremely challenging. One possibility, which we discuss in the new discussion section, is that TG2 modifies histone at specific sites, thereby facilitating chromatin access (7). Histone modification is notoriously difficult to analyze by mass spectrometry. The strategy used in the Nature paper was “facilitated” by the fact that this modification is quite wide-spread in serotonin expressing cells. In our case, the modification could be different than serotonylation, possibly on a different histone that H3K4me3, and specific to only a few promoters. In conclusion, the identification of the TG2 substrate, that eventually leads to the induction of GLUT-1 and GLUT-3, represents a research project of its own, which, if we were to solve it (and we plan to work on this, as we now know that we are dealing with a quite unique situation), would require to make two separate papers.

2.The other key point is related to the TG2-dependent post-translational modification of the GFPT. They have excluded that histaminylation or serotonylation take place on glutamine residues and in the discussion claim that "occasionally" they detected some glutamine deamination, however the important question of how TG2 post-translationally modify GFPT remains unsolved. This is a key question to be addressed before publication.

We repeated the immunoprecipitation of GFPT after ionomycin treatment, followed by mass-spectrometry analysis, that we had attempted already once in the initial revision. Again, we could not find evidence of serotonylation nor histaminylation. We observed deamidation, but in a pattern that was quite different from what we had observed in the first experiment, so we believe this reaction might occur “randomly” during sample preparation.

We agree that it is frustrating not to be able to describe this modification and we have plans to pursue this quest. We started working on obtaining a stable cell line with epitope tagged GFPT to increase purification yield and thus mass spectrometry depth. But as I previously pointed out before, looking for the unknown by mass spectrometry is very challenging, and this question might keep us busy for some years.

Let me finish by reminding you how many new discoveries are reported in our manuscript:

What was known	What we discovered
Nothing known regarding a role for TG2 in *Chlamydia* infection, and, more generally only very few piece of data on TG2 activation in bacteria or viral infection	- *C. trachomatis* infection induces an increase in the expression of TG2 and activates its transamidase activity - TG2 activity is required for chlamydial growth
C. trachomatis needs glucose and infection triggers an increase in the transcription of glucose transporter genes	- TG2 plays a central role in the transcriptional control of GLUT-1 and GLUT-3 - The transamidase activity of TG2 and HIF1 are required for the increase in the transcription of glucose transporter genes
Several TG2 targets have been identified, but no systematic proteomic approach in a physiological context has been reported	- We identify 62 potential TG2 substrates. While discovered in the context of *C. trachomatis* infection, many of these proteins are expected to be TG2 substrates in other contexts (e.g. we uncovered several known TG2 targets)
Nothing known regarding a link between TG2 activity and the hexosamine biosynthesis pathway	- We identified GFPT as a TG2 substrate and identified GFPT Q328 as prominent glutamine for modification by TG2 - We showed that GFPT modification by TG2 enhanced the production of UDP-NAcetylglucosamine
Nothing known about the *Chlamydia* need for UDP-GlcNAc	- We discovered that UDP-GlcNAc is highjacked by the bacteria - We discovered that UDP-GlcNAc, or an intermediate in the hexosamine biosynthesis pathway, is required for bacterial growth, and, more specifically, to sustain bacterial division.

I look forward to your feedback on the new version of our manuscript.

1. Sharma, M., Machuy, N., Bohme, L., Karunakaran, K., Maurer, A. P., Meyer, T. F., and Rudel, T. (2011) HIF-1alpha is involved in mediating apoptosis resistance to *Chlamydia trachomatis*-infected cells. *Cell Microbiol* **13**, 1573-1585
2. Kumar, S., Donti, T. R., Agnihotri, N., and Mehta, K. (2014) Transglutaminase 2 reprogramming of glucose metabolism in mammary epithelial cells via activation of inflammatory signaling pathways. *Int. J. Cancer* **134**, 2798-2807

3. Rossin, F., D'Eletto, M., Macdonald, D., Farrace, M. G., and Piacentini, M. (2012) TG2 transamidating activity acts as a reostat controlling the interplay between apoptosis and autophagy. *Amino Acids* **42**, 1793-1802
4. Filiano, A. J., Bailey, C. D., Tucholski, J., Gundemir, S., and Johnson, G. V. (2008) Transglutaminase 2 protects against ischemic insult, interacts with HIF1beta, and attenuates HIF1 signaling. *FASEB J.* **22**, 2662-2675
5. Kumar, S., and Mehta, K. (2012) Tissue transglutaminase constitutively activates HIF-1alpha promoter and nuclear factor-kappaB via a non-canonical pathway. *PLoS One* **7**, e49321
6. Gundemir, S., Colak, G., Feola, J., Blouin, R., and Johnson, G. V. (2013) Transglutaminase 2 facilitates or ameliorates HIF signaling and ischemic cell death depending on its conformation and localization. *Biochim. Biophys. Acta* **1833**, 1-10
7. Farrelly, L. A., Thompson, R. E., Zhao, S., Lepack, A. E., Lyu, Y., Bhanu, N. V., Zhang, B., Loh, Y.-H. E., Ramakrishnan, A., Vadodaria, K. C., Heard, K. J., Erikson, G., Nakadai, T., Bastle, R. M., Lukasak, B. J., Zebroski, H., Alenina, N., Bader, M., Berton, O., Roeder, R. G., Molina, H., Gage, F. H., Shen, L., Garcia, B. A., Li, H., Muir, T. W., and Maze, I. (2019) Histone serotonylation is a permissive modification that enhances TFIID binding to H3K4me3. *Nature* **567**, 535-539

2nd Editorial Decision 30th July 2019

Thank you for submitting a revised version of your manuscript and please accept my apologies for the delay in getting back to you with our decision due to detailed discussions within the team. Your study has been sent back to the original referees and their reviews are enclosed below.

As you will see, while referee #2 and #3 find that their criticisms have been sufficiently addressed and recommend the manuscript for publication, referee #1 remarks that two major points raised in his/her comments require further investigation. In particular, this reviewer feels that the new experiments do not conclusively address how TG2 regulates the transcription of glucose transporters in infected cells and the underlying transcriptional mechanisms. Furthermore, s/he stresses that how TG2 post-translationally modifies GFPT remains unresolved.

We understand the technical limitations in identifying the nature of GFPT modification. However, as indicated in the previous decision letter, we agree with referee #1 that solving these issues is essential for publication in The EMBO Journal. Given that the revised manuscript does not sufficiently address all the referee points and our policy allows only one single round of revision, I am afraid that we are unable to offer publication of your work at this stage.

However, given the clear relevance of your data to the more immediate field, I would be happy to reconsider a revised version of your manuscript that addresses the remaining points from referee #1. The revised manuscript will be assessed by the same referee and we will need strong support from him/her.

****

REFEREE REPORTS

Referee #1:

The new version of the paper entitled "nfection-driven activation of transglutaminase 2 boosts glucose uptake and hexosamine biosynthesis" by Agathe Subtil et al. is substantially improved upon revision, however the authors did not address the two major points I raised in my comments. I think these points need to be clarified in order to render this manuscript suited for publications in EMBO Journal.
1. The authors have performed an experiment in which, by silencing HIF-1alpha, observed a reduction in GLUT-1 and GLUT-3 transcription. This is not a direct evidence of the fact that this is the pathway regulated by TG2. The authors must demonstrate in which way does TG2 regulate/modify this transcriptional factor during the bacterial infection.
2.The other key point is related to the TG2-dependent post-translational modification of the GFPT. They have excluded that histaminylation or serotonylation take place on glutamine residues and in the discussion claim that "occasionally" they detected some glutamine deamination, however the important question of how TG2 post-translationally modify GFPT remains unsolved. This is a key question to be addressed before publication.

Referee #2:

We have considered the revised manuscript and found that the authors have responded to the majority of the issues raised by us (and certainly the most significant points). Thus, at this point, I would recommend that the revised manuscript be accepted.

Referee #3:

I thank the authors for considering most of my suggestions. They have addressed adequately my major concern, and also my minor concerns. I think this is an excellent manuscript and work. I have no further remarks.

Appeal received - authors' response 2nd August 2019

I am deeply surprised by your decision on our revised manuscript. I was aware of the "one revision round" policy of The Embo Journal. I, as many authors, support this policy, which clarifies expectations, and reduces publication delays. However, as authors, we understand that the policy works both ways: the editor will not introduce novel requests as to his/her expectations after the revision is submitted.

You had clearly stated in your initial decision letter what your expectations were: "In particular, referee #1 asks you to clarify how TG2 regulates the transcription of glucose transporters in infected cells and the underlying transcriptional mechanisms. Referee #2 requests you to show how bacteria increase TG2 expression and to test the specificity of CP4d inhibitor. Referee #3 asks you to compare the effects of TG2 inhibition and knockdown on bacterial size/cell division." The characterization of the modification of GFPT by TG2 was not among these requests. How can it now become a requisite for acceptation?

I also respectfully disagree with the conclusion that we failed to answer reviewer #1's question regarding the transcriptional mechanism of GLUT1/3 up-regulation, as it had been initially formulated. I copy below his/her comments:

1. The authors show that, in the presence of the TG2 inhibitor CP4d or TG2 KO cells, the transcription of the glucose transporter genes was no longer induced by the infection, thus indicating that TG2 is necessary for the control of GLUT-1 and GLUT-3 transcription. In line with this assumption they should demonstrate how TG2 regulates the transcription of these glucose transporters. The study proposes that the TG2-dependent constitutive NF-kB activation by promoting the expression of the HIF in turn might enhance GLUT-1 transcription. It well known that TG2 can regulate gene expression by modifying many key transcriptional factors such as SP1 and HSF1 and recently TFIID via the serotonylation of H3K4me3. Thus, in order to demonstrate the role of TG2 in the Chlamidia infection it is important to explain which transcriptional mechanisms is involved in the induction of the expression of these genes.

Reviewer #1 had rightly pointed out that different transcription factors had been shown to be regulated by TG2, and listed the best known. In the case of Chlamydia infection, we proved during revision that HIF-1a was necessary for the TG2 regulated transcriptional up-regulation of GLUT1/3. The molecular mechanism between TG2 expression and HIF-1a upregulation, which is the object of the novel comment by reviewer #1, has been studied in details by the group of Dr. Metha and is the object of a whole publication. I attach this paper, which is cited several times in our manuscript. Moreover, the transcriptional regulation mechanism is a very secondary question in our story, which as you know is centered on TG2 transamidating activity and its targets. I sincerely don't understand how this minor point, which is covered by the existing literature, became a reason not to accept a manuscript that contained a lot of novel information of broad significance, as acknowledged by all 3 reviewers.

I thank you in advance for your time and cooperation.

Appeal Editorial Decision 8th August 2019

Thank you for contacting us regarding our decision on your manuscript. I have now re-discussed your manuscript in detail with our chief-editor Bernd Pulverer (cc'ed here) and the other members of the editorial team. Our offer remains to consider a fully revised manuscript for the EMBO Journal and further that our sister journal EMBO reports would publish the present dataset. We would also like to give you more insight into the rationale for our decision.

First, I reassure you that we considered all the referees' reports and carefully evaluated the new

data. Addressing the mechanism of TG2-mediated upregulation of GLUT-1 and GLUT-3 is a key issue raised by both referee #1 and #2, as indicated in the decision letter. While we appreciate that you show that knockdown of HIF-1a reduces the transcriptional up-regulation of GLUT-1 and GLUT-3 upon Chlamydia infection, we agree with referee #1 that the specificity of HIF-1a in transcriptional expression of GLUT1/3 in your system remains unclear. Furthermore, Metha et al. demonstrate that TG2 acts through NF-kB to constitutively induce the transcription of HIF-1a and that this function does not require the TG activity. These findings appear to substantially differ from your model.

Given that your study focuses on TG2 transamidation activity and its targets, in our view the transcriptional mechanism(s) whereby TG2 regulates GLUT1/3 expression is an important point that needs to be duly addressed for the manuscript to be published in The EMBO Journal. Please note that referee #1's concerns about how TG2 post-translationally modifies GFPT, although per se well taken, was not in our view a reason for rejection. Thus, we did not introduce any new issues and instead only ask you to conclusively address the referee's original request.

We trust this explains the rational of our decision. I reiterate our offer to consider a substantially revised manuscript that would show the mechanism of TG2-HIF-1a-mediated regulation of GLUT1/3.

Appeal Editorial Decision 8th January 2020

Thank you for submitting a revised version of your manuscript. It has now been seen by referee#1, who finds that the remaining criticisms have been sufficiently addressed and recommends the manuscript for publication.

However, before we can formally accept the manuscript there are a few editorial issues concerning text and figures that I need you to address.

REFEREE REPORTS

Referee #1:

The last version of the ms including the new data obtained using the TG2 -/- MEFs with constitutive expression of wild type TG2, or of a point-mutant of TG2 that has no transamidase activity (C277S mutant (3)) have added interesting informations to the paper. Although the authors were unable to answer to all my previous requests I think the ms is now acceptable for publications on EMBO Journal.

2nd Revision - authors' response 21st January 2020

The Authors have made the requested editorial changes.

Accepted 31st January 2020

I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Agathe SUBTIL
Journal Submitted to: The EMBO Journal
Manuscript Number: 2019-102166

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 iustified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Each experiment was repeated 3 or more times to ensure adequate power.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The number of animals used is made visible on the figure (one dot/animal)
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No animal was excluded
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
Is there an estimate of variation within each group of data?	Results are represented in dot plots, so the variability can be appreciated immediately

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

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).	Yes
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	This has been reported

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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.	Done
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Done
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.	I confirm compliance with ARRIVE guidelines

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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F- Data Accessibility

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