EMBO reports

TG2 regulates the heat shock response by the post-translational modification of HSF1

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 10 October 2017

Thank you for the submission of your research manuscript to our journal.

We have now received a complete set of reviews from all referees, which I include below for your information. The referee reports are quite in agreement with each other. It appears that all referees consider the reported findings exciting and novel but all referees also point out that the data are currently not fully convincing and not sufficient to support the proposed model. In particular the data on CF patient cells need to be expanded, it remains unclear how a cytosolic Hsp70 can affect protein quality control in the ER and if the status of the mutant CFTR is altered at all to name a few of the concerns.

The concerns are numerous and it is clear that a major revision would be required before potential publication in EMBO reports. On the other hand, given the potential interest of your findings and also based on your feedback to the referee reports, I would like to give you the opportunity to address the concerns and would be willing to consider a revised manuscript with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board.

Should you decide to embark on such a revision, acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will 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. Please let us

know if you need more time for the revision so that we can extend the deadline accordingly. I should however point out that we will reassess novelty if revisions are received more than six months from the date when the initial decision letter was sent, which would be April 10th, 2018 in your case.

Supplementary/additional data: Up to five figures can be submitted in the Expanded View format. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

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

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

REFEREE REPORTS

Referee #1:

The heat shock transcription factors (HSFs), and especially HSF1, which is the most studied member of the mammalian HSF family, is best known as the transcriptional regulator of the protective processes against protein-damaging or proteotoxic stress insults and pathologies associated with protein aggregation, such as neurodegenerative diseases. On the other hand, HSF1 is frequently overexpressed and constitutively activated in various cancers. Beyond the classical heat shock genes, HSF1 is now known to regulate a great number of non-canonical target genes and it is obvious that HSF1 function depends on the context, including the upstream signaling pathways and various interacting partner proteins. In addition, HSF1 undergoes complex post-translational modifications during its activation-deactivation cycle. In this manuscript, the authors report a new control mechanism, by Transglutaminase 2 (TG2), for HSF1 activation. Using TG2 KO mice and MEFs derived thereof, Rossin and co-workers show that induction of Hsp70 (HSPA1/B) is defect upon exposure to proteotoxic stress. They show that overexpression of TG in the KO background rescues the Hsp70 induction. Furthermore, they propose that in the absence of TG2, HSF1 is unable to mount the heat shock response due to its inability to form trimers and localize to the nucleus. The physiological impact of TG2-HSF2 interaction is demonstrated by investigating samples from patients of cystic fibrosis (CF). Although the topic of this study is of great interest and the original observation is well described (Figures 1 and 2), there are a number of shortcomings, which need to be eliminated before the results are solid and qualify for a publication. Below, I summarize the most urgent concerns.

1. The physiological/functional relevance is not adequately demonstrated. While the authors show that the samples from CF patients treated with the TG2 inhibitor cysteamine display decreased expression of Hsp70, there is no evidence for a regulation of the HSPA1A/B-HSF1 pathway by TG2 in CF pathogenesis. The results solely indicate that TG2 activity is needed for the efficient induction of Hsp70 upon stress (and possibly in disease). There are no results showing that the disruption of HSF1 activity and consequently the decrease in Hsp70 levels would lead to the positive effect in patients or in the cells derived from patients.

Importantly, the status of CFTR is not investigated at all in this study. This is particularly confusing and disturbing, since the authors state that the disease stems from inability of CFTR to dissociate from HSPA1A/B, which causes CFTR to be retained in the ER. To date, and to the best of my knowledge, there is no evidence for HSF1 to be able to regulate ER-resident HSPs. Therefore, it remains totally unclear how the dysregulation of cytoplasmic HSPs, by HSF1, would affect the

protein quality control in the ER. The model presented in Figure EV5 lacks experimental evidence and cannot be supported by the results included in this manuscript.

Although the authors present convincing evidence for improper HSPA1A/B upregulation upon stress in TG2-deficient cells, the results do not justify the conclusions presented in the title. In addition to Hsp70 (HSPA1A/B), many more proteins are induced by HSF1 upon stress and examples of them should be included in the analyses before the term "heat shock response" can be collectively used in the title.

2. Other conclusions are also weakly supported by the results shown in the manuscript. The authors state that HSF1 in TG2 KO MEFs is unable to efficiently translocate to the nucleus. Although there is clearly less nuclear HSF1 in TG2 KO MEFS exposed to stress (Figure 3B), HSF1 cannot be detected in the cytosolic extracts either in WT or KO cells. What happens to the cytosolic HSF1 in TG2 KO MEFs? Furthermore, the authors should show if their fractions are not contaminated by other cellular compartments by including more controls, for example, a Lamin A/C blot in panels A and C and a Tubulin blot in panels B and C of Figure 3.

3. On page 6, it reads that "the amount of hyperphosphorylated HSF1, shortly after heat shock, was reduced in TG2-/- MEFs as compared to TG2+/+ MEFs (Fig. 3E)". However, based on this figure, the amount of HSF1 is decreased rather than the hyperphosphortylated form of HSF1. For this purpose, the authors should use phospho-specific HSF1 antibodies. The problematic issue of HSF1 phosphorylation status and trimerization is evident also in Figure 5C, where the authors conclude that the phosphorylation of HSF1 is required for its trimerization in vitro. In general, it is assumed HSF1 is capable of trimerizing in vitro as a recombinant protein. However, several conditions, such as temperature, concentration and phosphorylation affect the kinetics of trimerization. Thus, it is necessary to investigate in more detail how TG2 impacts the thermosensing ability of HSF1.

4. In Figure 4, the results on TG2-HSF1 interaction are shown; by immunoprecipitation (panels A and B) and immunofluorescence (panel C). Unfortunately, the quality of immunofluorescence images is so poor that neither interaction nor co-localization can be assessed. Using PLA or FRET would help to capture more clearly possible interaction between TG2 and HSF1 in the nucleus of stressed cells. Also the quality of panels A and B is inadequate to support the conclusions presented in the manuscript. The authors may try different antibodies to obtain better results, and they could also use HSF1 and/or TG2 down-regulation to further strengthen the analyses of interaction between these proteins. Loading controls for the input samples should be added.

5. In the present Figure 5D, the ChIP results only from stressed cells are shown. It would be important to include the ChIP results also from non-stressed cells.

6. The results shown in Figure 6 are not clear. How was the densitometric analysis performed? In panels A and C, there is a clear difference in the levels of HSPs in the CFTR mutant samples treated with cysteamine, which is not reflected by the error bar on the graph. Given that n=4 (panel A) and n=2 (panel C), one would expect more variation. The authors should show all the samples so that the reader can get a better idea about various samples. In general, more patients should be analyzed to increase the number of samples. It would be also important to blot for HSF1 in panels A and B.

7. The conclusions drawn from the molecular modeling (Figure EV4 are too bold and should be softened throughout the manuscript.

8. Throughout the manuscript, the writing is sloppy and there are a lot of typos.

Referee #2:

Review of EMBO manuscript number: EMBOR-2017-45067-T

Title: TG2 regulates the heat shock response by the post-translational modification of HSF1

Herein, Rossin et al. posit that the enzyme transglutaminase 2 (TG2) aids proteostasis through a novel mechanism involving the post-transcriptional regulation of HSF1. The authors utilize

transgenic mouse tissue and primary mouse embryonic fibroblasts (MEFs) to reveal that TG2 helps activate HSF1 during heat stress and is required for the induction of the canonical heat-shock target, HSP70. Immunoprecipitation of either TG2 or HSF1 reveals that these two proteins form complexes in both the cytosol and nucleus. TG2 activity is also required for the robust formation of the activated HSF1 trimer following heat shock. Furthermore, the authors constitute that the specific PDI activity of TG2 is responsible for its effects on HSF1. As the activity of TG2 has been implicated in the pathogensis of cystic fibrosis (CF), the authors suggest that inhibiting TG2 PDI function with the small molecule cysteamine restores protein homeostasis in this disease model.

Overall, the findings are exciting that TG2 can regulate HSF1 activation under heat stress and of interest to the proteostasis field. However, there are still several questions which remain. First, they implicate HSF1 which is historically linked with cytosolic proteostasis regulation but provide analysis on a secretory substrate in CFTR. It is not necessary but it would strengthen the manuscript if they could show improved folding/function of a cytosolic substrate. Along those same lines, it would be interesting if this group could provide some insight into the activity of the ER unfolded protein response (erUPR) or ER associated degradation (ERAD). Both process have been extensively linked with quality control of CFTR and it might help explain their effects on CFTR. As there is emerging data that HSF1 activity in the cytosol can influence the UPR in the ER.

More specially, the authors utilize the HSPA1A, an inducible cytosolic HSP70, as a translational marker for HSF1 activity in whole mouse tissues and MEFs. They reveal that HSP70 induction in multiple organs, including the brain, lung, liver, and testis, is dependent on TG2 (Fig. 1). The authors show n=2 for western data in Fig. 1, and n=3 for the densitometry analysis. It is unclear if the displayed western data was used for densitometry or other data, and in general these n-values are concerning. The authors also do not report specific P-values in the text, which is preferable to simply giving a range. Furthermore, the authors do not discuss the apparent delayed activation of HSP70 in certain tissues in TG2-/- mice (at 3h), which may indicate that HSF1 may still become transcriptionally active, but at a much slower rate. Indeed the authors utilize shorter time points for the remainder of the paper.

Moreover, the authors describe the poor proteostatic conditions resulting in defective CFTR folding and maturation, and utilize cysteamine to show a rescue of this observation. In CFTRF508del cells, the authors show increased HSP70, increased HSP40, and increased HSF1 polymers (Fig 6). Treatment with cysteamine recovers these levels to CFTRWT levels. However, it remains unclear if cysteamine is restoring poor protein homeostasis in CFTRF508del cells versus inhibiting the ability of the cell to respond to proteotoxic stress.

Concern that should be addressed in the text or discussion:
1. The authors should cite the original work, rather than the review (Pirkkala et al., 2001). Furthermore, the original work revealed HSF1 in the nucleus under basal conditions, and increased levels following HS, slightly disagreeing with the data presented in this manuscript (no HSF1 in nucleus under basal conditions).
2. Are the IP - westerns performed in Fig 3 and Fig 4 done in the presence or absence of reducing agent?
3. Fig 3D densitometry: I am not convinced that the P-values indicated for MG1232 4h are correct with such large SEM error bars and n=3.
4. While the IP data is convincing, the colocalization of HSF1 and TG2 shown by immunofluorescence in Fig 4C is unconvincing.
5. Fig 6A: While only n=2 western blot data are shown, the n=4 densitometry data do not agree with the (shown) western data. Treatment with cysteamine appears to have variable results (as seen on the western), but that is not reflected in the error bars. Please include all western data as supplement.
6. The authors fail to support an observation in the results section "the regulation of HSF1-HSP70 pathway by TG2 occurs through its PDI activity and not the transaminating one." The authors only show that HSF1 trimerization by TG2 was interrupted in the presence of calcium. They give no data or evidence that the PDI activity over the transaminating activity is required, only making assumptions. Their reference treatment with Z-DON (Fig EV2C) does not affect nuclear transportation of HSF1 and use this as evidence that the transaminating domain has no effect on HSF1. However, the authors already show that HSF1 has no problem translocating to the nucleus in TG2-/- cells (Fig 3A-D). Furthermore, the ChIP data do NOT confirm the hypothesis as the authors state.

a. The authors MUST perform an in vitro HSF-1 polymerization assay in the presence of Z-DON to confirm this hypothesis.
b. Alternatively, the authors could mutate catalytic residues affecting PDI or transaminating activity on TG2 and then perform ChIP and/or polymerization assays.
7. The authors should perform enzymatic assays to assess the effects of cysteamine on TG2 catalytic activity.

While the authors reveal an interesting regulatory mechanism in which TG2 regulates HSF1 trimer formation and transcriptional activity, multiple spelling and grammatical errors, in addition to confusing sentence structures, mark this manuscript as unacceptable for publication in its current form. Fixing these errors and cleaning up the writing would provide additional confidence to the work performed herein. In conjunction with these corrections, addressing the western blot/densitometry issues mentioned above and further examination into the relevant catalytic activity of TG2 would reflect adequate quality for publication.

Referee #3:

This manuscript provides a novel regulatory pathway linking Transglutaminase TG2 with the master regulator of the heat stress-responsive pathway, the transcription factor HSF1 (Heat Shock Factor 1), with important pathological impact in Cystic fibrosis. This exciting finding is however not sufficient to allow publication in EMBO Reports in the present state of the manuscript, which needs major revision.

Major comments:

Figure 4C:
- DAPI would be necessary to detect the nucleus. Pearson coefficient for confocal analysis should be used to quantify HSF1 and TG2 colocalization. Signal intensity is lower in CTR panels than in HS panels. Please comment.
- Can HSF1 and TG2 be co-immunoprecipitated?
Figure 6:
- There are discrepancies between the WB shown in Fig 6A and the quantification plots shown below. 1) the two F508del CFTR lane show similar HSP70 levels compared to the WT lanes ; 2) There are great differences in terms of HSP70 levels between the two lanes of F508del CFTR cells treated ex vivo by cysteamine (do they represent extreme situations among F508del CFTR patients?). Since 4 different patients were analyzed, why not show all of them? 3) What are the TG2 levels in F508del CFTR versus WT cells, and before or after ex vivo treatment (there is a need for such data that would be the equivalent of the ones shown for in vivo treatment in Fig. EV3)?
- Figure 6D: How many patients were used for the quantification of HSF1 polymer amounts? n=4 as in 6B?
- HSF1 levels are poorly informative in terms of HSF1 activity. HSF1 phosphorylation of Serine-326 (HSF1-P at S326) is a good marker of HSF1 activation and should be used (as in Roth et al. PloS Biol, 2014, for example; PMID: 25406061; DOI: 10.1371/journal.pbio.1001998). This would add an important proof for the reduction in HSF1 polymer levels in F508del CFTR patient cells treated with cysteamine.
- The decrease in HSP70 levels in F508del CFTR patient cells treated with cysteamine could lead to normalization of constitutive pathological stress conditions in patients, linked to proteostasis imbalance due to the F508del CFTR protein (putatively linked to reduction in the amount of HSF1 polymeric forms). Is the HS response (or response to any other relevant stress that activated the HSF pathway) impaired in the F508del CFTR cells? Does this cysteamine-induced normalization of the HSF pathway allow better induction of the HS response?
- What is the interpretation of the decrease in the HSF pathway in F508del CFTR cells? is only it a hallmark of reduction in TG2 activity? Does it affects the F508del CFTR levels or subcellular localization? Is it interpreted as a sign of proteostasis rescue?
- In that sense, do inhibitors of HSF1 would ameliorate the status of F508del CFTR cells (triptolide, Au et al. "Identification of inhibitors of HSF1 functional activity by high-content target-based screening". 2009, J Biomol Screen 14: 1165-1175. PMID: 19820069. DOI: 10.1177/1087057109347472; see Neef also et al., "Heat shock transcription factor 1 as a therapeutic target in neurodegenerative diseases". Nat Rev Drug Discov. 2011 10:930-44. PMID: 22129991;

and Roth 2014 cited by the Authors)? Would it also affect TG2 levels?
- In the F508del CFTR cells, ChIP of HSF1 to the Hsp70 promoter would be informative, if feasible in patient cells. In any case, Hsp70 mRNA levels should be shown, before and after cysteamine treatment.
- (p7) The authors say that that "Both patients, [...] showed functional rescue of mutant CFTR protein after 4 weeks of in vivo therapy". What do they mean? How was this rescue measured and characterized, molecularly speaking?
- Could a model for CF mice (like one mimicking the F508del CFTR mutation) be treated by cysteamine to investigate its in vivo effects? Could such model be crossed with the TG2-/- mice as another (genetic) proof of the effect of counteracting TG2 activity and its role of the HSF pathway?
- Could the silencing of Hsf1, or Hsp70/Hsp40 mRNA in mimick cysteamine effect?

More general comments:
1- Why do the authors limit their exploration of the HS response to HSP70 (and in the last Figure to HSP40)? Are HSP25/27 affected as well, and HSP90 (see Roth et al. 2014)? Whether these actors are co-regulated in the same manner to HSP70 would strengthen the impact of TG2 on the HSF pathway. This is also of importance since their deregulation ao-is expected to affect proteostasis in CF patients.

2- There is a need for a positive control for TG2 activity.
3- Please precise mode of action of TG2 on the monomeric HSF1 structure and production of the active trimeric form:
- Although the authors refer to the work by Ahn & Thiele 2003 (and Lu et al., 2008) for the role of disulfide bonds in the activation of HSF1 (shift from intra-molecular within the monomeric form of HSF1 to intermolecular disulfide bonds between HSF1 monomers in the HSF1 trimeric form), the authors do not explain how the disulfide isomerase activity of TG2 acts on the HSF& monomer. A scheme presenting their hypothesis, at least, would be necessary. Molecular modeling would be of course appreciated.
- The schematic representation in FigEV5 is incorrect, because the monomeric form of HSF1 carries intramolecular S-S bonds between the HR/A-B and HR-C domains (Ahn and Thiele 2003). This has to be corrected and included in the discussion.
- About HSF1 activation and deactivation: Could TG2 be involved in the deactivation of HFS1 (back to the monomeric form through TG2 PDI activity?)
Minor comments:
- Figure 1: Since the authors base their paper on the transcriptional defects due to compromised HSF1 activation in the absence of TG2, they should show that the diminished levels in HSP70 protein reflect a decreased ability to induce Hsp70 mRNA levels. This data appears in the Figure 5E and would benefit from being put in Figure 1.
- In the "Results" part (paragraph "TG2 mediates HSF1 activation" p.5), the authors state that: "Activation of HSF1 requires a multi-step process that includes its inducible hyper-phosphorylation by several kinases ».
The authors should correct this sentence: indeed, global hyperphosphorylation of mammalian HSF1 accompany HSF1 activation, but is uncoupled with HSF1 activity activity (see Budzyński et al. « Uncoupling Stress-Inducible Phosphorylation of Heat Shock Factor 1 from Its Activation. » Mol Cell Biol. 2015 Jul;35(14):2530-40. doi: 10.1128/MCB.00816-14.).

- The following sentence is not clear: « where mutated CFTR, F508del (the most frequent mutation affecting CFTR), is unable to dissociate from HSP70 and hence retained in the endoplasmic reticulum, where it is rapidly degraded ». Can the authors explain how the cytoplasmic HSP70 can retain mutated CFRT in the ER?

Size and format of the manuscript.
Provided the authors would provide the above-indicated amendments, their manuscript might fit the format of a Scientific report.
- The manuscript as such comprises about 29 000 characters, which could even fit with the format of a Scientific report, provided Results and Discussion would be combined.
- The manuscript comprises 6 figures, which should easily be shortened to 5 Figures. For example, Figure 1 and 2 could be one single figure.
In conclusion, the manuscript presents an attractive, novel pathway linking HSF1 to TG2, with important pathological consequences in the devastating Cystic fibrosis pathology. However, in spite

of this novelty, the manuscript is far from meeting the standard of EMBO Reports, and major revision would be necessary to bring the manuscript to this level.

1st Revision - authors' response 24 January 2018

Referee # 1

- The physiological/functional relevance is not adequately demonstrated. While the authors show that the samples from CF patients treated with the TG2 inhibitor cysteamine display decreased expression of Hsp70, there is no evidence for a regulation of the HSPA1A/B-HSF1 pathway by TG2 in CF pathogenesis. The results solely indicate that TG2 activity is needed for the efficient induction of Hsp70 upon stress (and possibly in disease). There are no results showing that the disruption of HSF1 activity and consequently the decrease in Hsp70 levels would lead to the positive effect in patients or in the cells derived from patients.

Recent observations have confirmed that the overexpression of the Hsp40/70 system decreases the fraction of CFTR protein that achieves a functional fold (Yang et al., 1993; Liu et al., 2014), as well as the down regulation or inhibition of HSF1 leads to a partial rescue of F508del-CFTR cell surface channel activity (Roth et al., 2014). Here we demonstrate that the TG2, regulating HSP70-HSF1 pathway, favours F508del CFTR degradation. Indeed *Ex vivo* treatment of freshly brushed patient cells with cysteamine, a TG2 inhibitor, not only leads to a decrease in HSP70 expression and HSF1 activation (new Figure 7) but also increases the plasma membrane expression of the F508del CFTR protein (De Stefano et al., 2014; Tosco et al; 2016).
To validate the effect of TG2 ablation on CF, we added a set of new *in vivo* data on a novel mouse model in which we expressed the F508del CFTR mutation on a TG2 null background. We show that ablation of TG2 in a CF mouse model not only reduces HSP70 protein levels but also improves the disease phenotype restoring CFTR channel activity with reduced pulmonary inflammation as well as resistance to Pseudomonas aeruginosa infection (new Figure 6).

- To date, and to the best of my knowledge, there is no evidence for HSF1 to be able to regulate ER-resident HSPs. Therefore, it remains totally unclear how the deregulation of cytoplasmic HSPs, by HSF1, would affect the protein quality control in the ER. The model presented in Figure EV5 lacks experimental evidence and cannot be supported by the results included in this manuscript.

It is well known that either CFTR folding or mutant CFTR degradation requires cytoplasmic chaperones such as HSP70, HSP40, HSP27 (Yang et al., 1993; Kopito 1999; Lopes-Pacheco et al., 2015), all regulated by HSF1. Moreover, in the last years it has become clear that HSF1 is able to regulate not only cytosolic but also ER and mitochondria proteins (Takemori et al., 2006; Sakurai and Ota 2011; Heldens et al., 2011; Kim et al., 2017). We consider that the newly added data obtained by the CF mouse model (Figure 6) strongly support the conclusions summarized in Figure EV5.

- In addition to Hsp70 (HSPA1A/B), many more proteins are induced by HSF1 upon stress and examples of them should be included in the analyses before the term "heat shock response" can be collectively used in the title.

According to the Reviewer's suggestion, we analysed other target genes of HSF1 and we found that in absence of TG2 also the expression of HSP25 and BAG3, a co-chaperone of HSP70, is defective (Figure EV2D).

- The authors state that HSF1 in TG2 KO MEFs is unable to efficiently translocate to the nucleus. Although there is clearly less nuclear HSF1 in TG2 KO MEFS exposed to stress (Figure 3B), HSF1 cannot be detected in the cytosolic extracts either in WT or KO cells. What happens to the cytosolic HSF1 in TG2 KO MEFs?
Upon MG132 treatment the amount of HSF1 in the cytosol is higher in TG2–/– cells. Following the Reviewer’s consideration, we showed a new more representative WB also after HS (Figure 3A).

- The authors should show if their fractions are not contaminated by other cellular compartments by including more controls, for example, a Lamin A/C blot in panels A and C and a Tubulin blot in panels B and C of Figure 3.

Of course, we performed the cited controls in the nuclear and cytosolic fractions. Considering that the fractions are not contaminated since the protein bands of the marker in the WB images are undetectable, we considered to not include them in the final figures. However, a representative WB is shown below.

- On page 6, it reads that "the amount of hyperphosphorylated HSF1, shortly after heat shock, was reduced in TG2-/- MEFs as compared to TG2+/+ MEFs (Fig. 3E)". However, based on this figure, the amount of HSF1 is decreased rather than the hyperphosphortylated form of HSF1. For this purpose, the authors should use phospho-specific HSF1 antibodies. The problematic issue of HSF1 phosphorylation status and trimerization is evident also in Figure 5C, where the authors conclude that the phosphorylation of HSF1 is required for its trimerization in vitro. In general, it is assumed HSF1 is capable of trimerizing in vitro as a recombinant protein. However, several conditions, such as temperature, concentration and phosphorylation affect the kinetics of trimerization. Thus, it is necessary to investigate in more detail how TG2 impacts the thermosensing ability of HSF1.

As suggested by the Reviewer, we used a specific antibody that recognizes phosphorylation at Ser326, a hallmark for HSF1 activation. Unfortunately, this antibody works only on human samples and there is no one available for mouse. However, as reported in the new version of Figure 7, we used it to analyse the phosphorylation of HSF1 in nasal epithelial cells obtained from CF patients and to confirm transcription factor activation (Figure 7E).

- Unfortunately, the quality of immunofluorescence images is so poor that neither interaction nor co-localization can be assessed. Using PLA or FRET would help to capture more clearly possible interaction between TG2 and HSF1 in the nucleus of stressed cells. Also the quality of panels A and B is inadequate to support the conclusions presented in the manuscript. The authors may try different antibodies to obtain better results, and they could also use HSF1 and/or TG2 down-regulation to further strengthen the analyses of interaction between these proteins. Loading controls for the input samples should be added.

According to the reviewer's suggestion, in Figure 4C and D we showed new immunofluorescence images with DAPI to detect nuclei and we have also calculated the Pearson coefficient to measure co-localization of HSF1 and TG2.
About the co-immunoprecipitation, we consider that the down-regulation of HSF1 and/or TG2 could affect the co-immunoprecipitation efficiency not adding information about the interaction.

- ChIP results only from stressed cells are shown. It would be important to include the ChIP results also from non-stressed cells.

ChIP assay was performed only after HS, because it is well known, and also visible in our results (Figure 3B and D), that in basal condition HSF1 is not present in the nucleus making it impossible to immunoprecipitate.

- In panels A and C, there is a clear difference in the levels of HSPs in the CFTR mutant samples treated with cysteamine, which is not reflected by the error bar on the graph. Given that n=4 (panel A) and n=2 (panel C), one would expect more variation. The authors should show all the samples so that the reader can get a better idea about various samples. In general, more patients should be analyzed to increase the number of samples. It would be also important to blot for HSF1 in panels A and B.

As suggested by the reviewer, we increased the number of patients (n=6) to analyse the expression of HSP70, HSP40 as well as the trimerization and phosphorylation of HSF1. These new data are reported in Figure 7C and D, EV4B.

- The conclusions drawn from the molecular modeling (Figure EV4) are too bold and should be softened throughout the manuscript.

The aim of the molecular model reported in Figure EV4 is just to show the capacity of cysteamine to bind to the cysteines involved in the redox status of the active site of the enzyme, thus suggesting that through this binding the cysteamine can impair the TG2's PDI activity as demonstrated by the substantial effect on the HSF1 trimerization in vivo and in vitro. Furthermore, we performed an in vitro HSF1 polymerization assay in the presence of cysteamine. In the image reported below it is possible to appreciate that cysteamine treatment decreases the polymerization of HSF1 (B).

Referee # 2

- they implicate HSF1 which is historically linked with cytosolic proteostasis regulation but provide analysis on a secretory substrate in CFTR. It is not necessary but it would strengthen the manuscript if they could show improved folding/function of a cytosolic substrate. Along those same lines, it would be interesting if this group could provide some insight into the activity of the ER unfolded protein response (erUPR) or ER associated degradation (ERAD). Both process have been extensively linked with quality control of CFTR and it might help explain their effects on CFTR. As there is emerging data that HSF1 activity in the cytosol can influence the UPR in the ER.

It is well known that either CFTR folding or mutant CFTR degradation requires cytoplasmic chaperones such as HSP70, HSP40, HSP27 (Yang et al., 1993; Kopito 1999; Lopes-Pacheco et al., 2015), all regulated by HSF1. Moreover, in the last years it has become clear that HSF1 is able to regulate not only cytosolic but also ER and mitochondria proteins (Takemori et al., 2006; Sakurai and Ota 2011; Heldens et al., 2011; Kim et al., 2017).

- The authors show n=2 for western data in Fig. 1, and n=3 for the densitometry analysis. It is unclear if the displayed western data was used for densitometry or other data, and in general these n-values are concerning.

The densitometric analysis is referred to three different experiments. The WB is representative as reported in the figure legend.

- Furthermore, the authors do not discuss the apparent delayed activation of HSP70 in certain tissues in TG2-/- mice (at 3h), which may indicate that HSF1 may still become transcriptionally active, but at a much slower rate. Indeed the authors utilize shorter time points for the remainder of the paper.

As underlined by the Reviewer only in lung and testis we detect a late and partial induction of HSP70. In addition, in MEFs lacking TG2 also after a prolonged HS, HSP70 induction never occurs and the cells start to undergo apoptosis (Figure EV1). At the moment there are not elements that can help to explain what happens at HSF1during recovery time in lung and testis.

- Moreover, the authors describe the poor proteostatic conditions resulting in defective CFTR folding and maturation, and utilize cysteamine to show a rescue of this observation. In CFTRF508del cells, the authors show increased HSP70, increased HSP40, and increased HSF1 polymers (Fig 6). Treatment with cysteamine recovers these levels to CFTRWT levels. However, it remains unclear if cysteamine is restoring poor protein homeostasis in CFTRF508del cells versus inhibiting the ability of the cell to respond to proteotoxic stress.

Cysteamine is able to increase the plasma membrane expression of the F508del CFTR protein either in CF patients or in CF primary nasal epithelial cells as recently published by De Stefano et al., 2014 and Tosco et al; 2016. Our results indicate that TG2 inhibition by cysteamine in these cells, results in a decreased activation of HSF1-HSP70 pathway (Figure 7, EV4B) favouring CFTR trafficking despite its proteosomal degradation.

- The authors should cite the original work, rather than the review (Pirkkala et al., 2001). Furthermore, the original work revealed HSF1 in the nucleus under basal conditions, and

increased levels following HS, slightly disagreeing with the data presented in this manuscript (no HSF1 in nucleus under basal conditions).

According to the reviewer's suggestion, we modified the citation (Baler et al., 1993). In this work HSF1 seems not to be present in the nucleus in basal conditions. About this concern, there are discrepancies in the literature. Probably it could depend on the cell type (Ahn and Thiele, 2016 showed no nuclear HSF1 in MEF cells) or a bit contamination in the nuclear fraction.

- Are the IP - westerns performed in Fig 3 and Fig 4 done in the presence or absence of reducing agent?

All IPs and WBs are performed in presence of reducing agent except for the analysis of HSF1 trimers reported in figure 5A and B, figure 7E.

- Fig 3D densitometry: I am not convinced that the P-values indicated for MG1232 4h are correct with such large SEM error bars and n=3.

As correctly suggested by the Reviewer, we checked the P-value and it was *P < 0.05.

- While the IP data is convincing, the colocalization of HSF1 and TG2 shown by immunofluorescence in Fig 4C is unconvincing.

As suggested by the Reviewer, we performed the immunofluorescence images with DAPI to detect nuclei and we calculated the Pearson coefficient to measure co-localization of HSF1 and TG2.

- Fig 6A: While only n=2 western blot data are shown, the n=4 densitometry data do not agree with the (shown) western data. Treatment with cysteamine appears to have variable results (as seen on the western), but that is not reflected in the error bars. Please include all western data as supplement.

As suggested by the reviewer, we increased the number of patients (n=6) to analyse the expression of HSP70, HSP40 as well as the trimerization and phosphorylation of HSF1. These new data are reported in Figure 7C-F.

- The authors only show that HSF1 trimerization by TG2 was interrupted in the presence of calcium. They give no data or evidence that the PDI activity over the transaminating activity is required, only making assumptions. Their reference treatment with Z-DON (Fig EV2C) does not affect nuclear transportation of HSF1 and use this as evidence that the transaminating domain has no effect on HSF1. However, the authors already show that HSF1 has no problem translocating to the nucleus in TG2-/- cells (Fig 3A-D). Furthermore, the ChIP data do NOT confirm the hypothesis as the authors state. The authors MUST perform an in vitro HSF-1 polymerization assay in the presence of Z-DON to confirm this hypothesis.
b. Alternatively, the authors could mutate catalytic residues affecting PDI or transaminating activity on TG2 and then perform ChIP and/or polymerization assays.

In figure EV2C we showed that Z-DON treatment does not affect the amount of HSF1 in the nucleus and has no effect on the induction of HSP70 expression. Instead, in TG2-/- cells there is a lack in HSP70 induction and a decreased translocation of HSF1. According to this, by ChIP, we measured the binding of HSF1 to HSP70 promoter and it occurs only where TG2 is present (Figure 5). However, as suggested by the Reviewer, we performed an in vitro HSF1 polymerization assay in the presence of Z-DON to confirm this hypothesis. In the image reported below it is possible to appreciate that the Z-DON does not affect the polymerization of HSF1 (A) while the cysteamine treatment decreases the polymerization of HSF1 (B).

- The authors should perform enzymatic assays to assess the effects of cysteamine on TG2 catalytic activity.

As suggested by the Reviewer, we performed the in vitro assay in presence of Cysteamine as depicted in the above reported figure.

Referee # 3

- DAPI would be necessary to detect the nucleus. Pearson coefficient for confocal analysis should be used to quantify HSF1 and TG2 colocalization. Signal intensity is lower in CTR panels than in HS panels. Please comment.

As suggested by the Reviewer, we performed immunofluorescence images with DAPI to detect nuclei and we calculated the Pearson coefficient to quantify the co-localization of HSF1 with TG2.

- Can HSF1 and TG2 be co-immunoprecipitated?
The co-immunoprecipitation of TG2 and HSF1 is shown in Figure 4A and B.

- There are discrepancies between the WB shown in Fig 6A and the quantification plots shown below. 1) the two F508del CFTR lane show similar HSP70 levels compared to the WT lanes ; 2) There are great differences in terms of HSP70 levels between the two lanes of F508del CFTR cells treated ex vivo by cysteamine (do they represent extreme situations among F508del CFTR patients?). Since 4 different patients were analyzed, why not show all of them? 3) What are the TG2 levels in F508del CFTR versus WT cells, and before or after ex vivo treatment (there is a need for such data that would be the equivalent of the ones shown for in vivo treatment in Fig. EV3)

As suggested by the reviewer, we increased the number of patients (n=6) to analyse the expression of HSP70, HSP40 as well as the trimerization and phosphorylation of HSF1. These new data are reported in Figure 7C-F. Accordingly we modified the related graphs. Moreover, we also analysed the levels of TG2 in CF patients treated *ex vivo* with cysteamine.

Figure 6D: How many patients were used for the quantification of HSF1 polymer amounts? n=4 as in 6B?

We increased the number of patients (n=6) and we updated the related graphs.

HSF1 levels are poorly informative in terms of HSF1 activity. HSF1 phosphorylation of Serine-326 (HSF1-P at S326) is a good marker of HSF1 activation and should be used (as in Roth et al. PloS Biol, 2014, for example; PMID: 25406061; DOI: 10.1371/journal.pbio.1001998). This would add an important proof for the reduction in HSF1 polymer levels in F508del CFTR patient cells treated with cysteamine.

According to the Reviewer's suggestion, we analysed the HSF1phosphorylation and it occurs mainly in CF patients and it is largely decreased by cysteamine (Figure 7E and F).

- The decrease in HSP70 levels in F508del CFTR patient cells treated with cysteamine could lead to normalization of constitutive pathological stress conditions in patients, linked to proteostasis imbalance due to the F508del CFTR protein (putatively linked to reduction in the amount of HSF1 polymeric forms). Is the HS response (or response to any other relevant stress that activated the HSF pathway) impaired in the F508del CFTR cells? Does this cysteamine-induced normalization of the HSF pathway allow better induction of the HS response?

It has recently been demonstrated that in bronchial epithelial cells from cystic fibrosis patients the heat shock response is up regulated (Roberts 2014; Roth et al., 2014). The authors found that triggering the HSR (by heat-shocking the cells) led to the rapid degradation of mutant CFTR protein. In these cells, the constitutively active form of HSF1 leads to overexpression of HSP70 exacerbating the mutant CFTR degradation. We confirmed these findings in nasal epithelial cells where HSP70 is upregulated together with HSP40 and HSP27 (Figure 7C and D, EV4B). As suggested by the reviewer probably cysteamine, affecting TG2 activity and consequently the HSF1 activation, normalizes HSF1 pathway, as revealed by a decreased protein levels in the HSF1 target chaperones.

- What is the interpretation of the decrease in the HSF pathway in F508del CFTR cells? is only it a hallmark of reduction in TG2 activity? Does it affects the F508del CFTR levels or subcellular localization? Is it interpreted as a sign of proteostasis rescue?

The analysis HSP70 expression and HSF1 activation was performed in nasal epithelial cells from CF patients treated *ex vivo* with cysteamine in which the plasma membrane expression of the F508del CFTR was confirmed as described in De Stefano et al., 2014 and Tosco et al; 2016. Moreover, we added a set of *in vivo* data on a novel CF mouse model in which we expressed the F508del CFTR mutation on a TG2 null background. We show that either the ablation of TG2 or its inhibition by cysteamine in a CF mouse model not only reduces HSP70 protein levels but also significantly restores CFTR channel activity.

In that sense, do inhibitors of HSF1 would ameliorate the status of F508del CFTR cells (triptolide, Au et al. "Identification of inhibitors of HSF1 functional activity by high-content target-based screening". 2009, J Biomol Screen 14: 1165-1175. PMID: 19820069. DOI: 10.1177/1087057109347472; see Neef also et al., "Heat shock transcription factor 1 as a therapeutic target in neurodegenerative diseases". Nat Rev Drug Discov. 2011 10:930-44. PMID: 22129991; and Roth 2014 cited by the Authors)? Would it also affect TG2 levels?

As already published by Roth et al., 2014, HSF1 inhibition leads to stabilization and trafficking of F508del-CFTR in bronchial epithelial cell line (CFBE cells). As suggested by the reviewer, we used a HSF1 inhibitor to analyse the effect on TG2 protein. We found that KRIBB11, a specific inhibitor of HSF1, leads to a decrease in TG2 protein levels after 48 hours in bronchial epithelial cells. Moreover, we confirmed in these cells an increase in F508del-CFTR trafficking (data not shown).

- In the F508del CFTR cells, ChIP of HSF1 to the Hsp70 promoter would be informative, if feasible in patient cells. In any case, Hsp70 mRNA levels should be shown, before and after cysteamine treatment.
Unfortunately, to perform ChIP in primary nasal cells is very difficult due to the high amount of sample required. However, according to the reviewer's suggestion, we analysed the mRNA levels of HSP70 in patient cells (see below).

- (p7) The authors say that that "Both patients, [...] showed functional rescue of mutant CFTR protein after 4 weeks of in vivo therapy". What do they mean? How was this rescue measured and characterized, molecularly speaking?

The analysis HSP70 expression was also performed in freshly isolated brushed nasal cells from CF patients treated *in vivo* with cysteamine in which the plasma membrane expression of the F508del CFTR was evaluated as described in De Stefano et al., 2014 and Tosco et al; 2016.

- Could a model for CF mice (like one mimicking the F508del CFTR mutation) be treated by cysteamine to investigate its in vivo effects? Could such model be crossed with the TG2-/- mice as another (genetic) proof of the effect of counteracting TG2 activity and its role of the HSF pathway?

According to the Reviewer's consideration, we added a set of *in vivo* data on a novel mouse model in which we expressed the F508del CFTR mutation on a TG2 null background. We show that ablation of TG2 in a CF mouse model not only reduces HSP70 protein levels but also improves the disease phenotype significantly restoring CFTR channel activity with reduced pulmonary inflammation as well as resistance to Pseudomonas aeruginosa infection (new Figure 6).

- Could the silencing of Hsf1, or Hsp70/Hsp40 mRNA in mimick cysteamine effect?

We agree with the reviewer that it would be interesting to evaluate the effect of silencing the HSP70/HSF1 pathway, however, in primary cells, the transfection efficiency is very low and to have a good rate of silencing is not possible. However, we believe that the experiment performed with the HSF1 inhibitor could of course mimick the silencing.

- Why do the authors limit their exploration of the HS response to HSP70 (and in the last Figure to HSP40)? Are HSP25/27 affected as well, and HSP90 (see Roth et al. 2014)? Whether

these actors are co-regulated in the same manner to HSP70 would strengthen the impact of TG2 on the HSF pathway. This is also of importance since their deregulation ao-is expected to affect proteostasis in CF patients.

According to the Reviewer's suggestion, we analysed other HSF1target genes and we found that in absence of TG2 also the expression of HSP25 and BAG3, a co-chaperone of HSP70, is defective (Figure EV2D). Moreover, we found that TG2 inhibition by cysteamine in nasal epithelia cells from CF patients, not only deregulates HSP70 but also HSP40 and HSP27 (new Figure 7D and EV4A) which are both involved in the CFTR proteostasis.

- There is a need for a positive control for TG2 activity

We tested the PDI activity of TG2 on HSF1 after HS and MG132 treatment and unfortunately we do not know any possible substrate of the enzyme (positive control?) under these conditions. Actually this is another important finding of this study, since HSF1 is the first soluble substrate for the TG2 PDI activity so far identified.

- Please precise mode of action of TG2 on the monomeric HSF1 structure and production of the active trimeric form:
Although the authors refer to the work by Ahn & Thiele 2003 (and Lu et al., 2008) for the role of disulfide bonds in the activation of HSF1 (shift from intra-molecular within the monomeric form of HSF1 to intermolecular disulfide bonds between HSF1 monomers in the HSF1 trimeric form), the authors do not explain how the disulfide isomerase activity of TG2 acts on the HSF& monomer. A scheme presenting their hypothesis, at least, would be necessary. Molecular modeling would be of course appreciated.

According to the reviewer, we modified the final model (Figure EV5) to explain the possible action of the PDI activity of TG2.

- The schematic representation in FigEV5 is incorrect, because the monomeric form of HSF1 carries intramolecular S-S bonds between the HR/A-B and HR-C domains (Ahn and Thiele 2003). This has to be corrected and included in the discussion.

According to the reviewer, we modified the final model (Figure EV5)

- About HSF1 activation and deactivation: Could TG2 be involved in the deactivation of HFS1 (back to the monomeric form through TG2 PDI activity?)

Of course it could be possible. According to this, its known that TG2 could be able to break disulphide bonds too (Hasegawa et al., 2003; Malorni et al., 2009; Mastroberardino et al., 2006).

- Figure 1: Since the authors base their paper on the transcriptional defects due to compromised HSF1 activation in the absence of TG2, they should show that the diminished levels in HSP70 protein reflect a decreased ability to induce Hsp70 mRNA levels. This data appears in the Figure 5E and would benefit from being put in Figure 1.

We thank the reviewer for the suggestion, but in figure 1 are showed the HSP70 protein levels in mice while the mRNA are referred to MEF cells analysed in the following figures and correlate with the ChIP analysis performed on HSP70 promoter.

- In the "Results" part (paragraph "TG2 mediates HSF1 activation" p.5), the authors state that: "Activation of HSF1 requires a multi-step process that includes its inducible hyper-phosphorylation by several kinases ».
The authors should correct this sentence: indeed, global hyperphosphorylation of mammalian HSF1 accompany HSF1 activation, but is uncoupled with HSF1 activity activity (see Budzyński et al. « Uncoupling Stress-Inducible Phosphorylation of Heat Shock Factor 1 from Its Activation. » Mol Cell Biol. 2015 Jul;35(14):2530-40. doi: 10.1128/MCB.00816-14.).

According to the reviewer's suggestion, we corrected the sentence.

- The following sentence is not clear: « where mutated CFTR, F508del (the most frequent mutation affecting CFTR), is unable to dissociate from HSP70 and hence retained in the endoplasmic reticulum, where it is rapidly degraded ». Can the authors explain how the cytoplasmic HSP70 can retain mutated CFRT in the ER?

According to the reviewer's suggestion, we modified the text to make it clearer.

2nd Editorial Decision 14 March 2018

Thank you for the submission of your revised manuscript to EMBO reports. I apologize for my delayed response but we have only recently received the final referee report. Unfortunately, former referee 3 was not available anymore and I have therefore asked referee 1 to also evaluate your response to the concerns of this referee.

As you will see, both referees are positive about the study and only request textual changes. Both, referee 1 and 2 remark that the findings should be better placed into the context of what is already known about the biology of HSF1. Therefore, please extend your discussion and also address discrepancies between your findings and earlier publications, as outlined by both referees. Please also discuss the role of HSF1 and ERAD in the context of CFTR turnover versus CFTR maturation through the Golgi to the plasma membrane.

Browsing through the manuscript myself, I noticed several minor editorial things that we require before we can proceed with the official acceptance of your manuscript:

- Your manuscript will be published as Article. Therefore, please include a separate Discussion section. This will also give you more space to discuss your findings in the context of earlier literature as outlined above.

- Our data editors have already checked the figure legends for completeness and I have also looked at the description of the statistical analysis. Please review the attached file with our comments. Please also see one comment in the methods section, regarding the TG2 and CFTR[F508del] mice.

- Please provide a callout for Figure 4D in the text. It is currently never mentioned.

- Please review the Author Checklist. Section C, point 7, e.g., has not been completed. Please note that this list will be published together with your manuscript.

- We noticed that some of the Western blots were contrast-modified rather strongly, e.g., HSP70 in Figure 1A. Please review these panels and provide images with less modification.

- Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (width x height). You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

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

REFEREE REPORTS

Referee #1:

The authors have substantially revised the manuscript according to the raised concerns.

The authors should, however, emphasize better that the HSF1 biology is complex and context dependent. The authors should appreciate the wealth of literature on the subcellular localization of this transcription factor in the presence and absence of various stimuli. It is well documented that

HSF1 can reside in the nucleus and also regulate gene expression without any detectable stress stimuli. It would be important to refer to adequate papers, published extensively by, for example, the Nakai laboratory. Therefore, it is difficult to comprehend the statement that "in basal condition HSF1 is not present in the nucleus making it impossible to immunoprecipitate". Please, explain this discrepancy between the results shown here and those published previously.

It is also important to refer to the original papers on the complex and multi-step regulation of HSF1 through multiple post-translational modifications, such as phosphorylation. As stated by several reviewers, the global hyperphosphorylation of mammalian HSF1 has been shown to be uncoupled with HSF1 activity (Budzynski et al. 2015), which should be included in the references. In the current text, the authors have not stated this properly.

Referee #2:

In this revised manuscript, Rossin et al. incorporate new data and modified text to address the reviewers concerns. They also present a point-by-point rebuttal to discuss every comment brought up by the reviewers. Overall, this group has adequately addressed several of the reviewers and this manuscript is closer to being publication ready. It is great that they were able to increase the CF patient numbers.

Some of the authors comments did not address the concerns brought up by all the reviewers. On several occasions, they just cited previous papers, which supported their results but neglected to mention papers that didn't support results. They rightfully cite extensive papers showing some overlap between ER UPR and Heat shock response. However, they neglect to include ChIP results from non-stressed cells saying, "in basal conditions HSF1 is not present in the nucleus making it impossible to immunoprecipitate". However, lung epithelia of CF patients are not undergoing constant heat stress and a fully activated HSF1 would not necessarily occur in these cells. Proteotoxic stress at its peak is not capable of fully activating HSF1 like heat. This is a big concern for the paper. Several emerging studies are implicated basal HSF1 function in the absence of heat including several cancer and aging studies. So yes, HSF1 is doing something without heat stress and this group should be aware of this information.

They did not address the first comment from reviewer 2 regarding the role of ERAD in this entire process. They merely copied and pasted the exact same response from referee one. This was an unsatisfactory response and did not even mention ER associated degradation which is a very widely accepted modulator of CFTR. Does HSF1 activate ERAD? Is ERAD active in this context?

2nd Revision - authors' response 24 March 2018

In this new revised version we have modified the manuscript following all your indications.
- As you suggested, we have included in the revised version of the manuscript the Discussion section in which we addressed the possible differences between our findings and the data reported in previous publications (as outlined by both referees). We also discussed the role of HSF1 and ERAD in the context of CFTR turnover versus CFTR maturation through the Golgi to the plasma membrane. Finally, as stated by the Reviewer 1, we discussed the role of the HSF1 phosphorylation in the context of its biological activity and we included in the References list the paper from Budzynski et al. (2015) as indicated by the Reviewer 1.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Mauro Piacentini
Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2017-45067V2

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)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	we performed all the experiments in triplicate at least
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	we used between 3 and 9 animals for condition and we performed statistical analysis
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
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.	Randomization based on a single sequence of random assignments.
For animal studies, include a statement about randomization even if no randomization was used.	Simple randomization was performed to prevent the selection of bias and insures against the accidental bias.
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	Blinding outcome assessment is performed to reduce the risk of bias of animal studies.
5. For every figure, are statistical tests justified as appropriate?	yes, they are.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	yes, we determined statistical significance using the Student's t-test or one-way ANOVA test.
Is there an estimate of variation within each group of data?	yes, there is.
Is the variance similar between the groups that are being statistically compared?	yes, it is.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

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

http://datadryad.org

http://figshare.com

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

http://biomodels.net/

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

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).	HSP70 (Enzo Life Science, ADI-SPA-820; AB_2039253); TG2 (Cell Signalling, 3557); mono and poly ubiquitinated conjugates (Enzo Life Science, BMI-PW8810); HSF1 (Enzo Life Science, ADI-SPA-901; AB_2039202); HSF1 (Millipore, ABE1044); HSP40 (Proteintech, 10838-1-AP; AB_2277491); phospho HSF1 (AbCam, AB47369); PARP (Enzo Life Science, BML-SA250; AB_2052271); Caspase 3 (Cell Signalling, 9662; AB_331439); BAG3 (Proteintech, 10599-1-AP; AB_2062602); Hsp25/27 (Enzo Life Science, ADI-SPA-801); Actin (Sigma, A-2066); Tubulin (Sigma, T-4026); Lamin A/C (Santa Cruz, SC376248)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	we used mouse embrionic fibroblats and primary nasal epithelial cellsfrom Cystic fibrosis patients. We performed PCR test to verify the absence of mycoplasma infection.

* 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.	C57Bl/6 mice WT and knock out for TG2. 129/FVB mice heterozygous for F508del mutation. All the procedures in mice were approved by the local Ethics Committee for Animal Welfare (IACUC No. 713) and were carried out in strict respect of European and National regulations.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Ethics Committee for Animal Welfare (IACUC No. 713)
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.	The ARRIVE guidelines are consulted.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	EudraCT number #2013-001258-82 approved by Local Ethics Committee, Protocol reference #85/13
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.	All patients gave written informed consent at the time of the clinical study. The experiments was performed according to the principles of WMA Declaration of Helsinki and the Department of Health and human Service Belmont Report.
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.	EudraCT number #2013-001258-82
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).	N/A
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.	no, it couldn't.