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Protein synthesis inhibition and GADD 34 control IFN-heterogenous expression in response to dsRNA

Alexandre Dalet, Rafael J Arguello, Alexis Combes, Lionel Spinelli, SÈbastien Jaeger, Mathieu Fallet, Thien-Phong Vu-Manh, Andreia Mendes, Jessica Perego, Marisa Reverendo, Voahirana Camosseto, Marc Dalod, Tobias Weil, Manuel Santos, Evelina Gatti, Philippe Pierre

Corresponding author: Philippe Pierre & Evelina Gatti, CIML-Centre d'Immunologie de Marseille Luminy

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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.)

Additional Correspondence 15 July 2016

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and the comments are provided below.

In this case I have decided to do a pre-consultation with you prior to taking the decision on the manuscript.

As you can see below, the referees raised a number of constructive concerns. One important point is that further in vivo and virus infection data is needed to support the physiological relevance of the key findings. Both referees #1 and 2 raise this point. This issue and the other raised points need to be resolved for consideration here. To extend the analysis along the lines indicated by the referees will require some efforts. Therefore before taking the decision in this case I would like to ask you to provide me with a point-by-point response of what you can do within 3-6 months to address the raised concerns. Based upon your response I will then take the decision on your manuscript.

REFEREE REPORTS

Referee #1

The paper " Ppp1r15a/GADD34 controls heterogeneity of IFN-β production in response to dsRNA" present a highly interesting hypothesis, that is that inhibition of translation serves as a danger signal which strongly enhances the activation of the IRF3/7 pathways. The demonstrate that the GADD34 transcription is controlled by IRF3/7, the model they present suggest that transfection with Poly IC leads to inhibition of translation via PKR activation, but also to induction of the GADD34 protein. However, I find that there is an inherent contradiction within the hypothesis presented by the authors that needs further explanation. How is the GADD34 mRNA induced by the IRF3/7 pathway and then translated (in condition where global translation is down) when IFNb is neither transcribed nor translated? Furthermore, several pathways exist that can induce IFNb synthesis while not inducing translational shutdown. While the idea of translational downregulation serving as a danger signal is novel, a series of additional control exp is required before publication.
In figure 1A the authors show that IFNb production requires GADD34 upon poly IC stimulation. The authors should investigate the need of GADD34 I a set of condition which will induce IFNb without inducing eIF2a phosphorylation. By transfection with the CARD domain of RIG-I, this will lead to a similar activation of IPS-1 induced signaling as poly II but without inhibiting translation. Secondly, they should induce IFNb by a IPS-1 independent route, either by treating cells with cGAMP or by transfecting with TRIF. Finally, the authors should use the constitutive active version of IFR3 to drive IFNb transcription. Does IFNb production depend upon GADD34 under these conditions?
Furthermore, they should isolate macrophages from GADD34 wt and -/- mice and test if IFNa and IFNb induction upon treatment with LPS or specific TLR 7 or 9 agonist depend upon PKR.

On page 11 the authors state "Thus, PKR likely exerts a transcriptional enhancing activity on IFN-ß and GADD34, mostly by reducing translation and potentially de novo synthesis of inhibitory molecules that could act directly on TBK1 or on one of its up-stream activator."
I think this claim is highly interesting but need to be backed by in vivo data using the PKR Ko mice and live virus infection.

In figure 3 the authors demonstrate that inhibition of protein synthesis promote IFNb mRNA induction. The authors should investigate the potential synergy of CHX mediated inhibition of protein with the above ways of inducing IFNb mRNA transcription.

In figure 4C why is there rather little difference between IFNb? Cells in non treated and poly IC treated cells.

In figure 5C the authors show that cells produce either IFNb or GADD34, in find this contradicting as both genes are regulated by the same transcription factors. Why do the burst in translation leading to synthesis of IFNb not lead to GADD34 synthesis ?

Figure 5 should be complemented by flow data using both a virus producing ample dsRNA and a virus like influenza which produces little dsRNA (maybe use Flu delta NS1). Staining for viral proteins or use of GFP labeled viruses will allow to discriminate between infected and uninfected cells. 2D flow for pyro and IFNb should be performed.
Several of the figures lack proper statistic and in other cases, f. eks fig 3B and C, SEM and mean are used on data from qPCR, this is inappropriate as qPCR data are rarely normal distributed (rather the Log to the data are normally distributed). Present dots for each exp and use non-parametric test.

I am not cable of evaluating the mathematical modeling, I hope a second reviewer with expertise in this field has been chosen.

Minor comment.
The names GADD34 Ppp1r15a are used randomly, this is confusing.

I think there is a consensus to call IPS-1 for MAVS.

The paper needs more proofreading.

Referee #2

In this manuscript the authors address the underlying mechanisms behind the apparently contradictory activities related to the induction of interferon (IFN) by double stranded RNA and an interferon mediated PKR-dependent inhibition of viral protein synthesis. They show that the GADD34 phosphatase which itself is induced in an IRF-3 dependent manner is essential not only for IFN production but also for relief of translational inhibition mediated by PKR. A mathematical model is proposed to support the notion that action of GADD34 licenses an alternation of protein synthesis and IFN production in individual cells.

General comment

Overall the authors present a well reasoned model to support their notion that exposure of cells to dsRNA induces a dynamic oscillation of translation that is linked to intensity of PKR phosphorylation (which remains stable) and the rate of degradation of GADD34. While the data strongly support the model the physiological relevance is more difficult to assess. The cells are transfected with dsRNA to mimic a virus infection but how closely the model would fit with such an infection is difficult to gauge. Also, while the signaling pathways activated by dsRNA transfection are well described, the authors do not take into consideration that they are likely activating other translational control pathways such as the 2'-5' oligadenylate-RNaseL pathway. There is also the issue of feedback following IFN-beta synthesis and the effect this might have (positive or negative) on the model.

Comments.

The authors need to repeat experiments on IFNAR -/- MEFs to determine whether a positive feedback loop is in play.

The role of the 2'-5' oligadenylate-RNaseL pathway on translational control needs to be taken into account.

Table 1 should be in supplementary information.

Referee #3

Review of "Ppp1r15a/GADD34 controls heterogeneity of IFN-β production in response to dsRNA" by Dalat et al.

Summary:
Dalat et al. pose and attempt to resolve an interesting paradox: how do cells infected with a virus both shut down translation to prevent viral propagation while at the same time mounting an IFN-β response that requires translation? The translational block is mediated by activation of the eIF2α kinase PKR, while IFN-β induction is mediated through RLR/IRF signaling. During ER stress, in which a different eIF2α kinase is activated (PERK), the transient translation block is relieved by feedback through induction of the PP1 phosphatase regulatory subunit GADD34. PERK activation leads to induction of the mRNA encoding GADD34 (Ppp1r15a) through production of the transcription factor ATF4, which is preferentially translated when eIF2α is phosphorylated and global translation is reduced. Surprisingly, the authors show here that following stimulation with a dsRNA mimic that models viral infection, GADD34 can be produced independently of PKR and ATF4 as part of the IRF3 regulon (downstream of RLR/IPS-1 activation), provided that translation is inhibited. Using flow cytometry to capture snapshots of cell populations over time following stimulation with the dsRNA mimic, the authors provide evidence that the heterogeneous induction of IFN-β across a population of cells is the result of only a subset of cells having translation activity, and that this non-uniform behavior is a consequence of GADD34 induction. Finally, using a mathematical model to simulate the signaling pathways they defined experimentally, they recapitulate the variable translation in individual cells that give rise to the population level heterogeneity in IFN-β production that has long been observed.

Comments on the experimental section:

Overall, I found the paper to be well framed, reasoned and executed. The major criticism I have is that the evidence for oscillation in the ability of cells to produce IFN-β is scant. The interpretation that individual cells oscillate is derived from static measurements of populations of cells, and thus no single cell is ever shown to transit the complete cycle, let alone oscillate. Granting the authors the benefit of the doubt that individual cells do transit the full cycle as depicted in Figure 5B, observing cells returning to the origin following a single cycle is adaptation, not oscillation: there is no evidence that the WT MEFs matched to GADD34ΔC MEFs ever go through the cycle more than once, and they do not follow the populations of WT MEFs matched to the irf3/7-/- MEFs long enough to show the completion of a second cycle.

The most surprising findings in the paper concern how different the effects of activating PKR and PERK are, despite the fact that they are both eIF2α kinases. First, while activation of PKR and PERK both lead to eIF2α phosphorylation, translation inhibition and subsequent induction of GADD34, the mechanism by which GADD34 is induced is different in response to dsRNA than in response to ER stress (the former requiring RLR/IRF and the latter requiring ATF4). Second, ISRIB - a drug that bypasses the effect of eIF2α kinases to maintain translation even when eIF2α is phosphorylated - can compensate for the loss of GADD34 in response to ER stress but not during stimulation with dsRNA mimic. The experiments demonstrating these results are well done and convincing, but the explanations the authors offer to explain these findings are rather unsatisfying. In particular, I could not follow the argument for why ISRIB synergizes with GADD34 in relieving stress granules following dsRNA treatment but cannot compensate for the lack of GADD34.

The major question left unaddressed in the paper is the mechanism by which translation inhibition is required for activation of the TBK1/IRF3 axis and transcriptional induction of IFNβ and GADD34. The authors speculate that the translation block leads to synthesis of an inhibitor, but I could not determine why they favor this hypothesis.

Comments on the mathematical model:
In general, the mathematical model is simple and elegant and provides both a high-level recapitulation of the experimental observations as well as theoretical underpinning. Rather than forcing agreement by increasing complexity, the authors chose to focus on the architectural principles to gain insight into broader phenomena. The use of the discrete time step framework allows for the random perturbation of parameters to more realistically simulate the behavior of individual cells. This is all laudable.

However, the authors missed an opportunity to abstract their model and position it in the larger context of the analysis of network motifs that foster particular behaviors such as adaptation and oscillations (see for example Milo et al. 2002 PMID 12399590). From my reading, the circuit they have described is a "repressilator" embedded in an incoherent feed forward loop (IFFL). (The IFFL is dsRNA both activating p-eIF2α via PKR and inhibiting it via GADD34; the "repressilator is p-eIF2α inhibiting translation which inhibits production of GADD34 which inhibits p-eIF2α.) Both of these network architectures are known to be able to drive oscillatory behaviors (Elowitz and Leibler 2000 PMID 10659856, Ma et al. 2009 PMID 19703401). An abstraction of Figure 6A that maps their specific molecular network onto these abstract motifs would make it obvious that they are describing a common network motif that can generate the behaviors they observe.

The one aspect of the model that I question is the requirement to simulate both transcription and translation of GADD34. It seems that the production of the functional protein is the only important part, particularly since both transcription and translation are exactly the same as both depend on the global translation rate with an identical delay (both = 1-Tl(t-d1)). While it is a fascinating experimental result that the Ppp1r15a mRNA is induced by translational inhibition in pkr-/- cells that are stimulated with dsRNA mimic, for the sake of the model, this detail seems unnecessary so long as the short-half-life protein is produced. Moreover, I also do not understand why the Ppp1r15a mRNA is seemingly lost following translation (=... - Tlg(t)*Gr(t-1)), as though productive engagement with the ribosome triggers its decay. Unless I am missing something, I would recommend removing equations 4 and 5 (add a constant time delay to the production of GADD34 if it is too fast without the transcription step).

Minor Points:
1. Throughout the text there are many instances of imperfect English and awkward phrasing too

numerous to delineate here. None of these impair the overall intelligibility of the text, but they detract from the overall readability. Copy editing by a native English speaker would be helpful.

2. To put the data in Figure 1A in context, it would be good to know how much IFNβ and GADD34 WT MEFs produce. I know the authors did this experiment previously (Clavarino et al. 2012), but I couldn't tell if the GADD34 overexpression was comparable.

3. In Figure 2C and D, why are the units for IFNβ different from GADD34?

4. Why are the p-eIF2α levels so high in basal conditions in the pkr-/- cells (Figure 3A)?

5. In Figure 3B, are the differences in induction GADD34 and IFNβ between WT and pkr-/- statistically significant?

6. In the florescence images in Figure 4A, it is difficult to see anything but the puro positive cells (the stress granules are barely visible).

7. In Figure 5, it appears that 10-20% of WT cells never arrest translation at all. I could not find an explanation of why these cells completely ignore the dsRNA signal.

8. In Figure 6B, if the simulation were run for several more cycles, are the oscillations damped out?

1st Editorial Decision 21 July 2016

Thanks for sending me your point-by-point response. I have now had a chance to take a look at it. I do appreciate the response and I would like to invite a revised manuscript. Please note that the revised manuscript will be sent back to the three referees and that I do need their support for publication here.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

1st Revision - authors' response 07 November 2016

Referee #1

The paper " Ppp1r15a/GADD34 controls heterogeneity of IFN-β production in response to dsRNA" present a highly interesting hypothesis, that is that inhibition of translation serves as a danger signal which strongly enhances the activation of the IRF3/7 pathways. The demonstrate that the GADD34 transcription is controlled by IRF3/7, the model they present suggest that transfection with Poly IC leads to inhibition of translation via PKR activation, but also to induction of the GADD34 protein. However, I find that there is an inherent contradiction within the hypothesis presented by the authors that needs further explanation. How is the GADD34 mRNA induced by the IRF3/7 pathway and then translated (in condition where global translation is down) when IFNb is ***neither transcribed*** *nor translated? Furthermore, several pathways exist that can induce IFNb*

synthesis while not inducing translational shutdown. While the idea of translational downregulation serving as a danger signal is novel, a series of additional control exp is required before publication.

Response: Reviewer 1 seems to have misunderstood an important point from our manuscript, since we clearly showed that both GADD34 and IFN-b mRNAs are transcriptionally co-regulated by IRF3 (Fig. 2B). Upon eIF2-a phosphorylation by PKR and global protein synthesis inhibition, only GADD34 mRNA can be efficiently translated, through a specific uORFs regulation system (see Lee et al., JBC (2009), jbc.M806735200), while IFN-beta mRNA is targeted to stress granules (Fig 5a). GADD34 accumulation reverts this process, re-establishing normal translation and allowing efficient IFN-beta synthesis and secretion. Therefore, there is no *inherent* contradiction within our hypothesis, since the particular mRNA translation regulation (always inverted compared to global protein synthesis activity) of short lived GADD34 drives most of the events described here.

In figure 1A the authors show that IFNb production requires GADD34 upon poly IC stimulation. The authors should investigate the need of GADD34 I a set of condition which will induce IFNb without inducing eIF2a phosphorylation. By transfection with the CARD domain of RIG-I, this will lead to a similar activation of IPS-1 induced signaling as poly II but without inhibiting translation. Secondly, they should induce IFNb by a IPS-1 independent route, either by treating cells with cGAMP or by transfecting with TRIF. Finally, the authors should use the constitutive active version of IFR3 to drive IFNb transcription. Does IFNb production depend upon GADD34 under these conditions?

Response: All our data indicates that GADD34 is only required for IFN-beta production upon induction of eIF2-a phosphorylation and global protein synthesis arrest. Any artificial system capable of inducing IFN-beta mRNA without affecting translation should therefore behave more or less normally, independently of GADD34, since it will not be translated efficiently in absence of protein synthesis inhibition, and conversely IFN-beta mRNA will be freely translated in the same conditions. This situation was already demonstrated by our result obtained with PKR -/- MEFs, as well as in GADD34-deficient GM-CSF bone marrow-derived dendritic cells (DCs), which do not arrest protein synthesis in response to poly (I:C) and still produce IFN-beta (Clavarino et al. PNAS, 2012). We have nevertheless address this point by stimulating STING with cGAMP in WT and PKR -/- MEFs and study the impact of artificial inhibition of protein synthesis with CHX on TBK1and IRF signaling (see point below).

Furthermore, they should isolate macrophages from GADD34 wt and -/- mice and test if IFNa and IFNb induction upon treatment with LPS or specific TLR 7 or 9 agonist depend upon PKR.

Response: As stated above, we have documented this point in Clavarino et al. PNAS 2012, showing that although IFN-beta and IL-6 mRNAs transcription is reduced in GADD34 -/- BM-DCs, no obvious impact on protein synthesis is observed in these cells. We therefore did not feel appropriate to include in the present work our previously published results.

On page 11 the authors state "Thus, PKR likely exerts a transcriptional enhancing activity on IFN-ß and GADD34, mostly by reducing translation and potentially de novo synthesis of inhibitory molecules that could act directly on TBK1 or on one of its up-stream activator."
I think this claim is highly interesting but need to be backed by in vivo data using the PKR Ko mice and live virus infection.

Response: We have now added to our manuscript, a biochemical demonstration that interference with protein synthesis directly impact the accumulation of negative regulators of the TBK1 pathways, including the de-ubiquitinase A20 and the phosphatase SHIP-1, which are both induced during the response to poly (I:C) (new Fig. 3).

We have also incorporated to the manuscript an entirely novel section describing how VSV infection impacts translation regulation in MEFs and the importance of GADD34, PKR and IFNAR to control viral replication (new Fig. 4). We further demonstrated that the enhancing effect of protein synthesis inhibition on TBK1 signaling also occurs during VSV infection (new Fig. 4). Given the time available for revisions and the complexity of performing infection experiments in vivo, we felt that such experiments will be more informative if first performed in vitro (new fig. 4). Indeed, viruses have many cellular targets and their impact and detection by PKR is extremely

variable, in particular with respect to immune cells infection (see Schultz et al. Cell Host Pathogens, 2010). It will take years to create of genetic models in PKR null background, in which protein synthesis can be manipulated at will, in time and cell or tissue specific manner, to be able to address this question accurately *in vivo*. Furthermore, impacting pharmacologically protein synthesis in live mouse was not an option, since in addition of affecting the general physiology of the animal, we will completely change the biochemistry of infected cells and non-infected cells, including immune cells that also contribute to anti-viral defenses. The first predictable effect of inhibiting globally translation will be to block type-I IFN release by activated pDCs, as well as preventing ISGs synthesis in non-infected cells, which will considerably change the conditions of viral dissemination.
it is, thus, very likely that experiments carried-out *in vivo* will not yield clear cut results and until appropriate animal models are generated, we will have to rely on our *in vitro* infection data (Fig. 4) that demonstrate that translation inhibition amplifies innate signaling in virally infected or dsRNA stimulated cells.

In figure 3 the authors demonstrate that inhibition of protein synthesis promote IFNb mRNA induction. The authors should investigate the potential synergy of CHX mediated inhibition of protein with the above ways of inducing IFNb mRNA transcription.

Response: We have addressed this issue using cGAMP and STING stimulation in MEFs in presence or absence of CHX (Fig. S2), and have demonstrated that artificial translation inhibition has also a strong potentiating effect on the TBK1/IRF3 signaling induced through this pathway.

In figure 4C why is there rather little difference between IFNb? Cells in non-treated and poly IC treated cells.

Response: In this figure (now 5C), **all cells** are treated **with poly I:C** and only ISRIB treatment varies. The difference observed with or without ISRIB is minimal, since we demonstrated that the drug is not sufficient to alleviate the requirement for GADD34 after pI:C detection. We modified the text to make the point clearer.

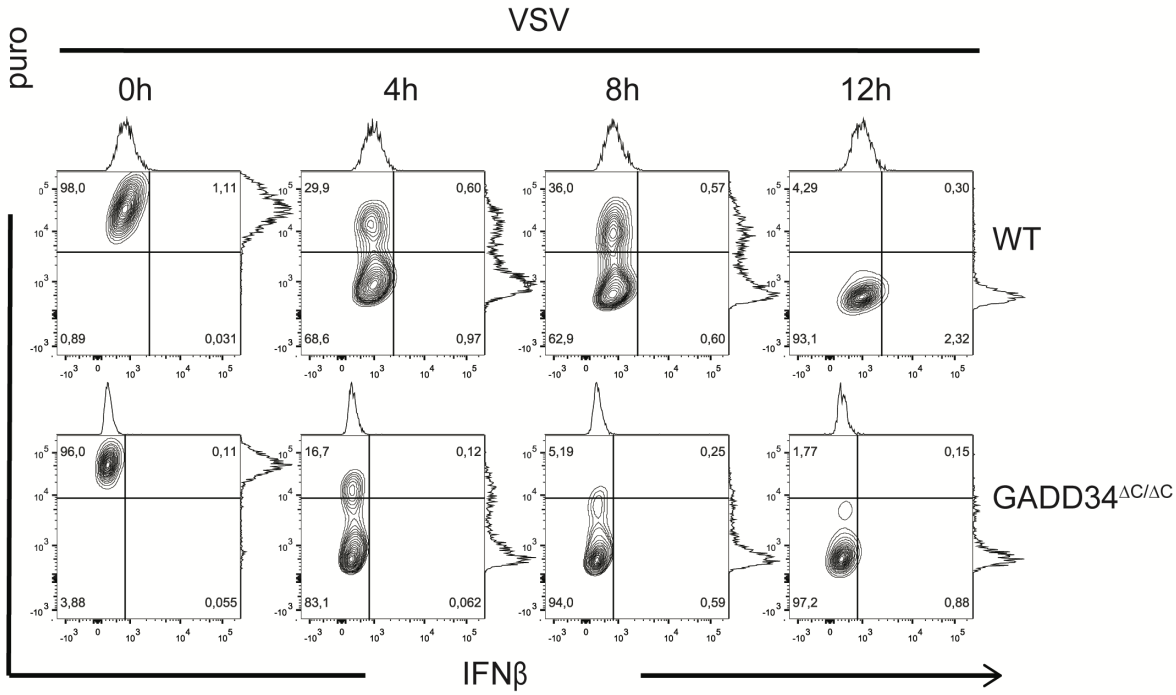
In figure 5C the authors show that cells produce either IFNb or GADD34, in find this contradicting as both genes are regulated by the same transcription factors. Why do the burst in translation leading to synthesis of IFNb not lead to GADD34 synthesis ?

Response: This is the entire point of our work, since the inverted mode of translation regulation of GADD34 (decoy ORFs regulation) and it's short half-life explain how GADD34 can accumulate upon general translation inhibition until its levels reach the threshold required to initiate p-eIF2-a dephosphorylation and to allow global protein synthesis re-activation and stress granules disassembly. In turn these two events lead to available IFN-beta mRNA translation and to the concomitant and rapid GADD34 protein disappearance, since the GADD34 mRNA is no longer translated in these conditions (high protein synthesis rate), explaining why GADD34 expression is no longer detected in high IFN producing cells. This extremely particular regulation of GADD34 is at the basis of the cycling model for translation regulation proposed at the end of the manuscript (Fig. 7).

Figure 5 should be complemented by flow data using both a virus producing ample dsRNA and a virus like influenza which produces little dsRNA (maybe use Flu delta NS1). Staining for viral proteins or use of GFP labeled viruses will allow to discriminate between infected and uninfected cells. 2D flow for pyro and IFNb should be performed.

Response: As stated above, our purpose was to define the potential of the host cell and why IFN-beta appears to be produced heterogeneously from cell to cell. We have now added experiments performed with Vesicular Stomatitis Virus (VSV) infected MEFs *in vitro* to complete our data obtained with poly I:C and explore the impact of translation inhibition on IRF3 signaling with a relevant pathogen. GFP-expressing VSV was chosen because of its sensitivity to type-I IFN and PKR expression. Most experiments performed with the live virus recapitulated our data obtained with poly I:C. However, although detectable by ELISA, albite to a lower level than with poly I:C (Fig. 4 and S2), VSV infection *in vitro* does not trigger sufficient type-I IFN levels to be able to detect its expression in individual cells by flow. We therefore did not include these data in the

manuscript, but provide to this reviewer a FACS plot for puro and IFN-beta, demonstrating how GADD34 deficiency impact infected cells in addition of the new Fig. 4.



Flow analysis of VSV infected WT and GADD34 deficient MEFs. Although type-I IFN is detectable by intracellular FACs in poly I:C treated WT cells, we could not visualize enough WT cells producing IFN upon VSV infection to be able to include these data in the manuscript. However, in GADD34 deficient cells, no trace of IFN could be detected by ELISA (Fig. 4) and translation arrest is more efficient across the MEFs population.

Several of the figures lack proper statistic and in other cases, f. eks fig 3B and C, SEM and mean are used on data from qPCR, this is inappropriate as qPCR data are rarely normal distributed (rather the Log to the data are normally distributed). Present dots for each exp and use non-parametric test.

Response: This has been addressed in Figures 2 and 3, and dot plots have been used instead of graphs.

I am not capable of evaluating the mathematical modeling, I hope a second reviewer with expertise in this field has been chosen.

Minor comment.
The names GADD34 Ppp1r15a are used randomly, this is confusing.
I think there is a consensus to call IPS-1 for MAVS.
The paper needs more proofreading.

Response: GADD34 has now been used throughout the text, except in some occasion requiring to specify the gene name (e.g transcriptomics and mathematical model). IPS-1 was replaced by MAVS all along the figures and text which was proofread.

Referee #2

In this manuscript the authors address the underlying mechanisms behind the apparently contradictory activities related to the induction of interferon (IFN) by double stranded RNA and an interferon mediated PKR-dependent inhibition of viral protein synthesis. They show that the GADD34 phosphatase which itself is induced in an IRF-3 dependent manner is essential not only for IFN production but also for relief of translational inhibition mediated by PKR. A mathematical

model is proposed to support the notion that action of GADD34 licenses an alternation of protein synthesis and IFN production in individual cells.

General comment

Overall the authors present a well reasoned model to support their notion that exposure of cells to dsRNA induces a dynamic oscillation of translation that is linked to intensity of PKR phosphorylation (which remains stable) and the rate of degradation of GADD34. While the data strongly support the model the physiological relevance is more difficult to assess. The cells are transfected with dsRNA to mimic a virus infection but how closely the model would fit with such an infection is difficult to gauge. Also, while the signaling pathways activated by dsRNA transfection are well described, the authors do not take into consideration that they are likely activating other translational control pathways such as the 2'-5' oligadenylate-RNaseL pathway. There is also the issue of feedback following IFN-beta synthesis and the effect this might have (positive or negative) on the model.

Comments.

The authors need to repeat experiments on IFNAR -/- MEFs to determine whether a positive feedback loop is in play.

Response: We know from the transcriptomic analysis of GADD34$^{\Delta C/\Delta C}$ MEFs stimulated with poly I:C, that the GADD34/PPP1R15a gene is transcribed in these conditions, despite minimal type-I IFN production in these cells. We have confirmed this observation by treating IRF3/7 KO MEFs with recombinant IFN-b and shown that GADD34 is not induced in these conditions (Fig. S1D). We nevertheless used IFNAR -/- MEFs to determine if the IFN feed-back loop impacts our experimental system and translation regulation (New Figures 4 and S2). We showed that IFNAR signaling is necessary to have a normal signaling response and to efficiently inhibit protein synthesis in all cells, presumably by maintaining the expression levels of RLRs and PKR (as in IRF3/IRF7 KO MEFs, see RIG-I blot Fig. S1D), however IFNAR is not required for the potentiation of TBK-1 and IRF3 signaling upon translation inhibition.

The role of the 2'-5' oligadenylate-RNaseL pathway on translational control needs to be taken into account.

Response: It is an interesting comment, however we did not take in account the 2'-5' oligoadenylate-RNaseL system in our model, because it is mostly activated upon type I-IFN exposure (ISGs) and therefore requires both time and active protein synthesis to be initiated. This is demonstrated by the fact the RNAseL is up-regulated in WT, but not GADD34 -/- MEFs (Fig. 1). Our experimental system aims at tracking the direct impact of dsRNA on protein synthesis inhibition and resolution by GADD34 in a relatively short time. It is therefore unlikely that the RNAseL system, which impacts mostly bystander cells or cells exposed to dsRNA for longer time, affects majorly our observations. Nevertheless, the results obtained in IFNAR -/- MEFs indicates that IFN signaling is required to have a normal response at the cell population level both transcriptionally and translationally, but individually IFNAR -/- MEFS can inhibit translation in response to dsRNA.

Table 1 should be in supplementary information.
Response: Table 1 is now in supplementary information.

Referee #3

Review of "Ppp1r15a/GADD34 controls heterogeneity of IFN-β production in response to dsRNA" by Dalat et al.

Summary:
Dalat et al. pose and attempt to resolve an interesting paradox: how do cells infected with a virus both shut down translation to prevent viral propagation while at the same time mounting an IFN-β response that requires translation? The translational block is mediated by activation of the eIF2α kinase PKR, while IFN-β induction is mediated through RLR/IRF signaling. During ER stress, in

which a different eIF2α kinase is activated (PERK), the transient translation block is relieved by feedback through induction of the PP1 phosphatase regulatory subunit GADD34. PERK activation leads to induction of the mRNA encoding GADD34 (Ppp1r15a) through production of the transcription factor ATF4, which is preferentially translated when eIF2α is phosphorylated and global translation is reduced. Surprisingly, the authors show here that following stimulation with a dsRNA mimic that models viral infection, GADD34 can be produced independently of PKR and ATF4 as part of the IRF3 regulon (downstream of RLR/IPS-1 activation), provided that translation is inhibited. Using flow cytometry to capture snapshots of cell populations over time following stimulation with the dsRNA mimic, the authors provide evidence that the heterogeneous induction of IFN-β across a population of cells is the result of only a subset of cells having translation activity, and that this non-uniform behavior is a consequence of GADD34 induction. Finally, using a mathematical model to simulate the signaling pathways they defined experimentally, they recapitulate the variable translation in individual cells that give rise to the population level heterogeneity in IFN-β production that has long been observed.

Comments on the experimental section:
Overall, I found the paper to be well framed, reasoned and executed. The major criticism I have is that the evidence for oscillation in the ability of cells to produce IFN-β is scant. The interpretation that individual cells oscillate is derived from static measurements of populations of cells, and thus no single cell is ever shown to transit the complete cycle, let alone oscillate. Granting the authors the benefit of the doubt that individual cells do transit the full cycle as depicted in Figure 5B, observing cells returning to the origin following a single cycle is adaptation, not oscillation: there is no evidence that the WT MEFs matched to GADD34ΔC MEFs ever go through the cycle more than once, and they do not follow the populations of WT MEFs matched to the irf3/7-/- MEFs long enough to show the completion of a second cycle.

Response: We agree with this reviewer and have been using the term “oscillation” mostly to illustrate GADD34 capacity of being alternatively made and destroyed in inverted correlation with translation activity. For the general behavior of the cells, we can probably use “cycling” rather than oscillating for our experimental work, although our modeling results in consecutive waves of protein synthesis activation and inactivation, closer to what can be called an oscillation. We have applied a more precise semantic in this reveised version of the manuscript, and use “adaptation” or “cycling” accordingly to the situation.

The most surprising findings in the paper concern how different the effects of activating PKR and PERK are, despite the fact that they are both eIF2α kinases. First, while activation of PKR and PERK both lead to eIF2α phosphorylation, translation inhibition and subsequent induction of GADD34, the mechanism by which GADD34 is induced is different in response to dsRNA than in response to ER stress (the former requiring RLR/IRF and the latter requiring ATF4). Second, ISRIB - a drug that bypasses the effect of eIF2α kinases to maintain translation even when eIF2α is phosphorylated - can compensate for the loss of GADD34 in response to ER stress but not during stimulation with dsRNA mimic. The experiments demonstrating these results are well done and convincing, but the explanations the authors offer to explain these findings are rather unsatisfying. In particular, I could not follow the argument for why ISRIB synergizes with GADD34 in relieving stress granules following dsRNA treatment but cannot compensate for the lack of GADD34.

Response: We know the eIF2-a phosphorylation is necessary for SG formation and it’s de-phosphorylation for SG dissociation. ISRIB acts by uncoupling eIF2B from the dominant negative effect of p-eIF2-a, therefore re-establishing translation initiation despite high level of PERK activity in the case of thapsigargin exposure. We observed in poly I:C exposed cells, in which PKR is the principal mediator of eIF2-a phosphorylation, that ISRIB has a small potentiating effect on SGs dissociation in WT cells, while this effect was absent from GADD34 -/- cells. Our explanation for this phenomenon is that GADD34 is required for an additional biochemical task that allows SGs dissociation in poly I:C stimulated cells, and that ISRIB by allowing eIF2B to function normally, adds up with this alternative GADD34 activity to accelerate SG dissolution and translation recovery in control cells. We are sorry that our explanations were unsatisfying and that the word “synergizes” was used, may be a bit lightly. We have removed this sentence, which in fact was not necessary to describe the ISRIB effect. Cleary, despite the novelty of this finding, the possible mechanistic explanations for this insensitivity to ISRIB are countless and will be the object of future research, since it is not the primary focus of our manuscript.

The major question left unaddressed in the paper is the mechanism by which translation inhibition is required for activation of the TBK1/IRF3 axis and transcriptional induction of IFNβ and GADD34. The authors speculate that the translation block leads to synthesis of an inhibitor, but I could not determine why they favor this hypothesis.

Response: Given the impact of translation inhibition on IkB expression (Fig. 3D) and its consequences on NF-kB signaling, It seemed logical that the same type of inhibitory system could exists for the TBK1/IRF3 pathway and that translation inhibition could reduce therefore the expression of different negative feed-back regulators of the IRF3 pathway. We have now shown that the expression of the de-ubiquitinase A20 and the phosphatase SHIP-1 are strongly affected by CHX treatment (new Fig. 3 and S2), indicating that several negative regulators of TBK1 phosphorylation are down-modulated during its activation by nucleic acids and this absence is likely to contribute to the potentiation of this signaling cascade.

Comments on the mathematical model:
In general, the mathematical model is simple and elegant and provides both a high-level recapitulation of the experimental observations as well as theoretical underpinning. Rather than forcing agreement by increasing complexity, the authors chose to focus on the architectural principles to gain insight into broader phenomena. The use of the discrete time step framework allows for the random perturbation of parameters to more realistically simulate the behavior of individual cells. ***This is all laudable.***

However, the authors missed an opportunity to abstract their model and position it in the larger context of the analysis of network motifs that foster particular behaviors such as adaptation and oscillations (see for example Milo et al. 2002 PMID 12399590). From my reading, the circuit they have described is a "repressilator" embedded in an incoherent feed forward loop (IFFL). (The IFFL is dsRNA both activating p-eIF2α via PKR and inhibiting it via GADD34; the "repressilator is p-eIF2α inhibiting translation which inhibits production of GADD34 which inhibits p-eIF2α.) Both of these network architectures are known to be able to drive oscillatory behaviors (Elowitz and Leibler 2000 PMID 10659856, Ma et al. 2009 PMID 19703401). An abstraction of Figure 6A that maps their specific molecular network onto these abstract motifs would make it obvious that they are describing a common network motif that can generate the behaviors they observe.

Response: We fully agree with the reviewer on that point and we thank him/her to point out the repressilator motif that was not known to our group. The consideration of network motives was raised since the beginning of the model design, because it immediately appeared that the presence of an IFFL will certainly lead to the presence of oscillations. The decision to do not bring that theoretical consideration in the article came from our desire to make the accessibility and comprehension of the model mechanism as easy as possible for a general biologist readership not necessarily used to mathematical models. However, this reviewer comments made us realized that we might have been too cautious and decided to introduce the explanation of the network motifs in the revised manuscript by a simple sentence leading to the detailed presentation in the methods.

Proposed edition of the text:
The sentences p20:

[...] Simulation of eIF2, GADD34 and global translation level dynamics for realistic parameter values demonstrate that oscillations can be intrinsically present in such model (Fig. 6B). Given the [...]

hasnow become:
p20
realistic parameter values demonstrate that oscillations can be intrinsically present in such model (Fig. 7B). Moreover, the model contains two network motifs (i) an Incoherent type-1 Feed-Forward Loop (IFFL) and (ii) a Repressilator, both of them described in the literature as generating oscillatory dynamics (see experimental procedure).

The one aspect of the model that I question is the requirement to simulate both transcription and translation of GADD34. It seems that the production of the functional protein is the only important

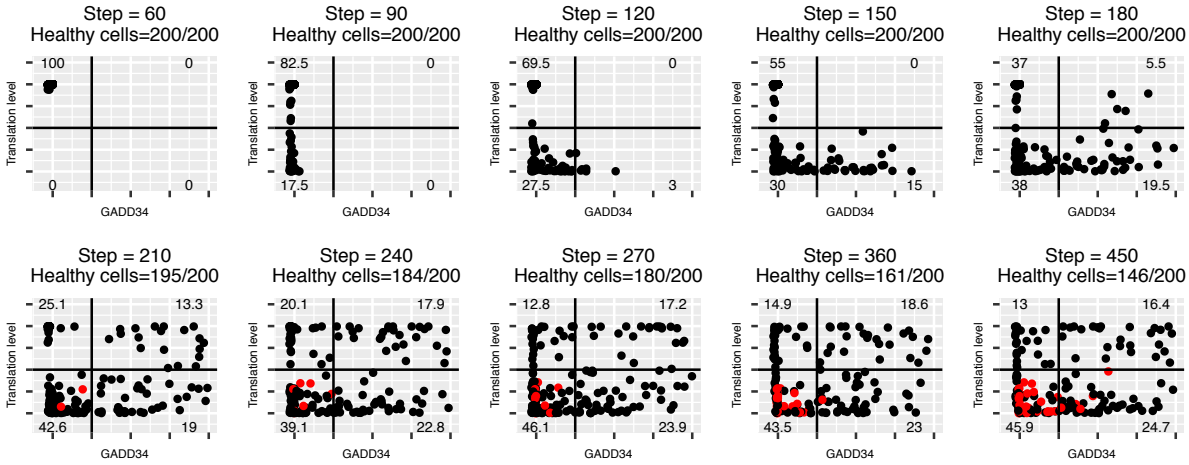
*part, particularly since both transcription and translation are exactly the same as both depend on the global translation rate with an identical delay (both = 1-Tl(t-d1)). While it is a fascinating experimental result that the Ppp1r15a mRNA is induced by translational inhibition in pkr-/- cells that are stimulated with dsRNA mimic, for the sake of the model, this detail seems unnecessary so long as the short-half-life protein is produced. Moreover, I also do not understand why the Ppp1r15a mRNA is seemingly lost following translation (=... - Tlg(t)*Gr(t-1)), as though productive engagement with the ribosome triggers its decay. Unless I am missing something, I would recommend removing equations 4 and 5 (add a constant time delay to the production of GADD34 if it is too fast without the transcription step).*

Response: We agree with the reviewer that mathematically strictly speaking, the presence of Ppp1r15a RNA may be not required and that it would be possible to conceive a simpler model without the equation (4) and (5), considering only the level of production of the GADD34 protein.

We produced this model with the following set of equations and included it in supplementary procedures:

1. P(t) = S1(Ds(t-1))
2. Tl(t) = S2(E(t-1))
3. Tlg(t) = 1-Tl(t-d1)
4. Gp(t) = Gp(t-1) + Tlg(t-d1) * C1 - C2
5. E(t) = C3 * P(t-1) - C4 * Gp(t-d2)

The standard behavior of the simplified model is similar as the complete model. When using the same parameters values just changing d1 from 30 to 20, the evolution of markers presented in figure 7B is almost the same (see below). Moreover, the simulation of virtual cells dynamics is also similar and do not present a qualitative difference with the result presented in the article (figure 7D). However, we think that keeping the presence of Ppp1r15a mRNA in the model is important for the comprehensiveness of the model. Again the model is designed to be accessible within biologist's conventional decoding frameworks and removing the equation describing GADD34 mRNA transcription is not in agreement with this concept and will bring confusion and potentially lack of consideration for this work.
We propose to introduce in a first step a model with the mRNA and then discuss in the supplementary method on the possibility to reduce the model to the set of equation presented above. Moreover, another point should be taken in consideration. In the model presented in the main text, the decay of Ppp1r15a mRNA is, as indicated by this referee, triggered by productive engagement in ribosomes. This model of mRNA decay was chosen because it is most simple and also supported by the literature (Roy and Jacobson, 2013, j.tig.2013.09.002.). Currently, we have no information on the way the Ppp1r15a mRNA decays and this is a point that suggests that conservation of the mRNA regulatory step in the model is important for potential evolution of the mathematical model, for example, what would be the influence of the decay model on the global model? We also think in future studies to improve the model by introducing the effect of stress granule on mRNA protection, those questions are obviously not trivia to addressed and modelled.



Minor Points:

1. *Throughout the text there are many instances of imperfect English and awkward phrasing too numerous to delineate here. None of these impair the overall intelligibility of the text, but they detract from the overall readability. Copy editing by a native English speaker would be helpful.*

Response: We had originally our manuscript edited by an American scientist, but have tried again to improve the overall quality of the text.

2. *To put the data in Figure 1A in context, it would be good to know how much IFNβ and GADD34 WT MEFs produce. I know the authors did this experiment previously (Clavarino et al. 2012), but I couldn't tell if the GADD34 overexpression was comparable.*

Response: Ectopically expressed GADD34 levels are comparable with the one presented in Clavarino et al. 2012 Figure 4E.

3. In Figure 2C and D, why are the units for IFNβ different from GADD34?

Response: These units heterogeneity was introduced during the last steps of the figures design and have now been corrected.

4. Why are the p-eIF2α levels so high in basal conditions in the pkr-/- cells (Figure 3A)?

Response: It seems that the absence of PKR in these MEFs is compensated by another eIF2K activity, we have often observed compensations effects in available strains of KO MEFs. For instance, the ATF4 -/- MEFs express higher level of GADD34 at steady state than WT (see Fig. 2B).

5. In Figure 3B, are the differences in induction GADD34 and IFNβ between WT and pkr-/- statistically significant?

Response: Missing statistics and significance have been added.

6. In the florescence images in Figure 4A, it is difficult to see anything but the puro positive cells (the stress granules are barely visible).

Response: We have improved the quality of the Immunofluorescence images in now Fig 5A, which had lost quality during the files conversion in PDF.

7. *In Figure 5, it appears that 10-20% of WT cells never arrest translation at all. I could not find an explanation of why these cells completely ignore the dsRNA signal.*

Response: Clearly even with high efficiency of pI:C transfection, we cannot be sure that all cells receive enough nucleic acid or express enough PKR to trigger translation arrest. Based on our results with IFNAR -/- and IRF3/7 -/- MEFs, insufficient expression RLR and PKR in infected or lipofected cells is likely to be responsible for this non-responding phenotype.

8. I*n Figure 6B, if the simulation were run for several more cycles, are the oscillations damped out?*

Response: In now figure 7B, the oscillations of the presented model are perfectly stable even when run for a long time (100x the presented time). In fact, the model produces a perfect cycle with no damping.

2nd Editorial Decision 29 November 2016

Thanks for sending me the revised manuscript. Your study has now been re-reviewed by the referees and their comments are provided below. As you can see from the reports, the referees appreciate the

introduced changes and support publication here. I would like to ask you to respond to the last few issues raised by referee #1 in the point-by-point response. But OK to go ahead and celebrate - congratulations on a nice study!!

REFEREE REPORTS

Referee #1:

The authors respond in length on the question raised by the reviewers. In general I believe that the authors are addressing many of the mechanistically question raised. However, I am concerned about the lack of in vivo evidence. Do the GADD34 deficient mice show any abnormalities in vivo in their IFN production as well as their overall ability to handle viral infections? And as raised by reviewer 3 (page 8 top), that authors failed to demonstrate that a cell go through the full cycle (I do not see the point in changing wording from oscillation to cycling)? While not fully convinced, I do not object to publication if my fellow reviewers are enthusiastic.

Referee #2:

The authors have responded to the reviews of the manuscript with written comments, appropriate changes in text and with the addition of new data to address specifc concerns.

Referee #3:

Dalet et al. have done an excellent job responding to the critiques. Their rebuttal letter was soundly argued, and the new data presented clearly speak to the major concerns. The inclusion of the VSV data, albeit in vitro, would seem to address the concerns about the physiological relevance of the pIC model that was brought up by the other reviewers.

They sufficiently addressed my semantic qualms and made helpful clarifications. I very much appreciate that they took the time to repeat the model simulations after removing the equations I suggested, and I am convinced by their rationale to retain these equations. Finally, the longer-term simulations do indeed predict sustained oscillatory behavior. It would be interesting to see if this is the case in cells!

2nd Revision - authors' response 12 December 2016

Referee #1:
The authors respond in length on the question raised by the reviewers. In general I believe that the authors are addressing many of the mechanistically question raised. However, I am concerned about the lack of in vivo evidence. Do the GADD34 deficient mice show any abnormalities in vivo in their IFN production as well as their overall ability to handle viral infections? And as raised by reviewer 3 (page 8 top), that authors failed to demonstrate that a cell go through the full cycle (I do not see the point in changing wording from oscillation to cycling)? While not fully convinced, I do not object to publication if my fellow reviewers are enthusiastic.

We thank reviewer 1 for his support. As mentioned in the introduction of the paper, we have already demonstrated the extreme susceptibility of GADD34-deficient neonate mice to Chikungunya virus infection (Clavarino et al., PLOS pathogens, 2012) and the reduced capacity of GADD34-deficient adult mice to mount type-I IFN response after poly I:C or Chikungunya virus challenge (Clavarino et al., PNAS, 2012), so there is little doubt that GADD34 and the therein described pathways are relevant to handle viral infections. Although the redundancy of the different host anti-viral systems to produce type-I IFN have co-evolved to deal with various virus type and viral countermeasures, the importance of the GADD34 pathway *in vivo* might change greatly according to the virus types and the host studied.

Concerning the full cycle "or not" of translation "ondulation", we show in Figure 6 E, that some cells can at least do 1 and 1/4 cycle (so at least 1 cycle, going towards 2 cycles). We agree however that the demonstration of a single cell performing several cycles would be more satisfactory, since it would validate further our mathematical model. We will try in our future work to find experimental conditions and design novel techniques to observe these multiple cycles, but in the meanwhile, we feel that the advances provided by our results, in term of single cell and protein synthesis measurement, bring about a completely novel conceptual framework to explore anti-viral signaling and type-I IFN production with a fresh eye.

Referee #2:
The authors have responded to the reviews of the manuscript with written comments, appropriate changes in text and with the addition of new data to address specifc concerns.

We thank reviewer 2 for his support and insightful comments.

Referee #3:

Dalet et al. have done an excellent job responding to the critiques. Their rebuttal letter was soundly argued, and the new data presented clearly speak to the major concerns. The inclusion of the VSV data, albeit in vitro, would seem to address the concerns about the physiological relevance of the pIC model that was brought up by the other reviewers.
They sufficiently addressed my semantic qualms and made helpful clarifications. I very much appreciate that they took the time to repeat the model simulations after removing the equations I suggested, and I am convinced by their rationale to retain these equations. Finally, the longer-term simulations do indeed predict sustained oscillatory behavior. It would be interesting to see if this is the case in cells!

We thank reviewer 3 for his support and encouraging comments about both our biological and mathematical work. We are indeed engaging ourselves on the hard path of multidisciplinarity, aiming at integrating mathematical modeling with experimental biology, and it would be extremely satisfactory to now demonstrate the existence of several oscillations in one cell. Although viral infection might not give enough time for the infected cells to undergo many cycles, and the techniques available to visualize translation arrest might not be ripped yet to perform such analysis with accuracy.

THE EMBO JOURNAL

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

Corresponding Author Name: Philippe PIERRE
Manuscript Number: EMBOJ-2016-95000R

Reporting Checklist For Life Sciences Articles

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 (see link list at top right).

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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- 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 (see link list at top right).

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
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- definitions of statistical methods and measures:
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  - 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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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11. Identify the committee(s) approving the study protocol.	Not applicable
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.	Not applicable
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not applicable
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