THE EMBO JOURNAL

Assembly and function of the amyloid-like translational repressor Rim4 is coupled with nutrient conditions

Diana Silvia Marina Ottoz, Lauren Tang, Annie Dyatel, Marko Jovanovic, and Luke Berchowitz
DOI: 10.15252/embj.2022113332

Corresponding author: Luke Berchowitz (leb2210@cumc.columbia.edu)

Review Timeline:

Submission Date:	19th Dec 22
Editorial Decision:	15th Feb 23
Revision Received:	18th Aug 23
Editorial Decision:	6th Sep 23
Revision Received:	20th Sep 23
Accepted:	25th Sep 23

Editor: Kelly Anderson

Transaction Report:

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

Dear Luke,

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by two referees whose comments are shown below.

Given the referees' positive remarks on the significance of your findings, I would like to invite you to submit a revised version of the manuscript, addressing the comments of the two reviewers. It is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. In this case, while the referees were fairly positive on some aspects, they do ask for a couple of more involved experiments to more fully support your model. Therefore, if you would prefer to leave these to another manuscript, I would alternatively be happy to discuss the suitability of your work with the EMBO reports editors who more often accept a more focused version. As a matter of practice, we typically discuss revision plans with authors and I am happy to do so in the coming weeks via zoom or email. I have also attached a guide for revisions to this email.

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: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact 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.

Yours sincerely,

Kelly

Kelly M Anderson, PhD
Editor
The EMBO Journal
k.anderson@embojournal.org

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (16th May 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

Referee #1:

Previous results provided evidence that yeast Rim4 is an RNA-binding protein that forms amyloid-like assemblies in meiosis and functions to repress the translation of certain meiotic genes in a manner requiring two of its three RRMs and its intrinsically disordered region (IDR), which is also necessary for assembly formation. These results suggested that translational repression depends on formation of Rim4 assemblies but how assembly formation promotes repression is not understood; nor was it understood how formation of Rim4 assemblies is controlled. In this report, the authors showed that Rim4 can form aggregates and repress reporter mRNAs harboring the 5'UTRs of two Rim4 target mRNAs when expressed ectopically in mitotic cells. This is significant because it eliminates an essential role for any other meiosis-specific factor at least for these two targets; although a third Rim4 target reporter mRNA was not repressed so that other factors could still be important for certain transcripts. They confirm the requirement for the Rim4 IDR for assembly and also show that the IDR is sufficient for assembly in mitotic cells. They provide evidence that RNA-binding by Rim4 is highly induced by the starvation conditions that induce sporulation and provide evidence that, unlike the RRMs, the IDR is dispensable for RNA binding; although the PNK assay they employed for RNA binding is rather indirect and does not necessarily reveal binding of authentic Rim4 mRNA targets. In addition, the evidence that the IDR is completely dispensable for RNA binding is somewhat questionable as it appears to be contradicted by the data presented in Supp. Fig.2D. Confirming that the IDR is required for reporter repression, they infer that assembly

formation is necessary for translational repression in a manner that goes beyond target mRNA binding per se. However, contrary to statements in the Abstract and Discussion, they do not elucidate the molecular mechanism of translational repression via Rim4 aggregates, which still remains unknown. They attempt to define the nutrient signals that trigger assembly formation and find that replacement of glucose with nonfermentable carbon sources in nitrogen-rich medium is sufficient to trigger assembly formation and reporter repression, whereas starvation for nitrogen permits partial assembly and reporter repression in the presence of glucose. These results are significant in suggesting that the absence of glucose is the nutrient signal that elicits Rim4 assembly and repression. Experiments using an analog-sensitive allele of TPK1 indicate that inhibiting PKA function is sufficient to trigger Rim4 assemblies in rich glucose medium. This should lead to Rim4-dependent reporter repression as well, but the relevant results were confounded by repression of the reporter mRNA in the absence of Rim4 on inhibition of PKA function, with little or no additional repression conferred on induction of Rim4. As an alternative approach, they showed that activation of PKA in starved cells by expressing the dominant RAS2Val allele eliminated Rim4 assemblies and seemed to abolish Rim4 repression of the reporter. This experiment (Fig. 3G) was also flawed however because the magnitude of Rim4 repression in the WT control cells was less 2-fold, compared to 5-fold or more in other experiments. This complication was coupled with unexpected induction of the reporter mRNA by RAS2Val in the absence of Rim4. Thus, while the evidence is strong that PKA impedes Rim4 assembly formation, it is not compelling that PKA also impairs Rim4 translational repression. Other experiments, possibly ones that avoid using the estradiol induced reporter to assay repression, would be required to bolster this key conclusion. They go on to provide evidence, obtained using anti-phospho antibodies that are presumably specific for PKA phosphosites, that the Rim4 IDR is phosphorylated by PKA in rich medium. Unfortunately, however, it was not established that PKA is required for IDR phosphorylation. Moreover, substituting 4 putative PKA sites in the IDR of chromosomal RIM4 with phosphomimetic Glu residues did not impair assembly formation or reporter repression in starved cells in the manner that would have been expected if PKA directly impairs Rim4 assembly by phosphorylating the IDR. Thus, it remains unclear how PKA impairs Rim4 assembly. They do find some evidence that the IDR 4-Ala substitution mutant slightly delays the disappearance of Rim4 protein and assemblies in the return to growth from sporulation/starvation conditions on addition of glucose, and also slightly impedes cell growth in this recovery phase; however, it was not convincing that the growth defect results from the persistence of Rim4 protein/aggregates because the growth delay appears many hours after there is no effect of the Ala mutations on Rim4 protein or assembly abundance.

In summary, it is both convincing and interesting that glucose is the most important nutrient that impedes Rim4 assembly and that PKA function is somehow involved in this. Important drawbacks however are that (i) the mechanism of PKA function in impeding Rim4 assemblies is unclear-it does not seem to involve direct Rim4-IDR phosphorylation-and (ii) the lack of compelling evidence that PKA function also blocks translational repression by Rim4. The evidence that Rim4 RNA-binding function is necessary but not sufficient for translational repression is promising, but there are concerns about the indirect nature of the PNK assay and the data obtained that provides evidence that eliminating the IDR had no effect on RNA binding, and corroborating evidence from RNA-IP is likely required to bolster this point.

Major comments:
-the PNK assay for RNA binding is not specific for Rim4 target mRNAs, nor it seems even for messenger RNA. It would be highly desirable to replace it with an RNA-IP assay (or even better RIP-Seq) to interrogate the effects of the RRM and IDR mutations on Rim4 binding to bone fide target mRNAs and try to confirm that the IDR is completely dispensable for binding such transcripts in starved cells. The large variance of the data for the IDR deletion shown in Fig. 2E underscores a shortcoming of this assay in establishing this key conclusion about the IDR in RNA binding by Rim4.
-The data in Fig. S2D on the IDR deletion variant seems to indicate a clear reduction in binding in the phosphoimage that cannot be explained by the anti-V5 (Rim4) immunoblot. These results would have to represent rather extreme outliers among replicates to be reconciled with the mean RNA/protein ratios shown in Fig. 2E for this variant. Also, statistical analysis is lacking for the results in panel E, making it unclear if the 3-rrm mutant truly has a reduced RNA/protein ratio.
-Additional experiments are required to show that inhibition of PKA in the tpk1-as strain or activation of PKA in the RAS2val strain alters translation of a Rim4 reporter in the predicted manner, overcoming the confounding effects of PKA function on the estradiol-induced reporter and the poor repression ratio for the WT cells in Fig. 3G.
-Additional text is required for the interpretation of the gels quantifying Rim4 assemblies. Fig. 3A gives the impression that the amount of Rim4 assemblies in N-starved cells grown with glucose are actually higher than those in rich medium with glycerol or acetate, but this is unlikely to be true considering that the overall amount of Rim4 on conventional SDS-PAGE doesn't vary for these conditions (Fig. S3A). To get a better estimate of the amounts of assemblies, they could quantify the proportion of total signal found at the top of the gel in the assembly bracket from replicate experiments. Presumably this would help overcome the high variability of cumulative signal throughout the lane, which can vary greatly but is expected to be nearly constant.
-fig. 2B: the data for the 3-rrm mutant here is unsatisfying as the total signal is unexpectedly very low; the same is true for the SPO+glucose condition in Fig. 3A.
-line 271: the claim that Rim4IDR(4A) protein levels were higher compared to wild type or Rim4IDR(4E) during sporulation is not supported by quantification of Western data from replicate experiments of the kind shown in Fig. 5A. If it is convincing merely from visual inspection of replicates, this should be stated and the replicates cited in the Source data, and the magnitude of the increase should be quantified from the replicates.
-line 293: the conclusion here is too strong as there is no direct evidence that PKA phosphorylates the Rim4 residues S525 and S612 implicated as sites of 14-3-3 protein binding in their previous paper, only a correlation between phosphorylation and PKA activity.
-the writing suffers overall in lacking explicit information that would assist the reading in grasping their data and interpretations of

results. There is insufficient citing of particular results in the text, especially for data shown in the Supplementary figures which are merely mentioned in passing. One gets the impression that the manuscript was prepared for publication in a journal with a much more restrictive character limit, preventing a "user-friendly" description of details of assays and key results obtained.
-lines 31-32: This claim in the Abstract is overstated. The mechanism for how PKA impedes Rim4 assembly is still unknown, as is the mechanism for how Rim4 assembly formation supports translational repression.

Other comments:
-should stipulate that "starvation conditions" always correspond to sporulation conditions
-the authors should note the confounding issue that the reporter mRNA is not induced in nitrogen starvation medium or SPO medium supplemented with Glucose (Fig. 3B). Is this understood?
-line 211: At odds with this statement, the induction of Rim4 does not appear inefficient in Fig. S3D.
-line 305: this is not true, as the role of assembly in repression is still a mystery

Referee #2:

This work from Ottoz et al provides interesting and important new insights into the way that yeast controls the movement of Rim4 into amyloid-like aggregates, and the concomitant Rim4 dependent repression of translation, during the transition into meiosis. Specifically, the authors show that the PKA kinase phosphorylates Rim4 in the presence of glucose and this blocks the assembly of Rim4 into insoluble aggregates. Then once cells inter starvation conditions, PKA is inhibited, driving the Rim4 dependent repression of translation.

The experiments presented are very well done and convincing up until the authors start to pick apart the role of individual phosphorylation sites in Fig. 4. Specifically, once the authors show that PKA controls the movement of Rim4 into aggregates and the Rim4 dependent repression of translation, they set out to determine which if any of the PKA sites on Rim4 drive these events. To do this they follow the phosphorylation of Rim4 in different conditions using an anti-PKA phosphosite antibody. They find that Rim4 reacts with this antibody in glucose, but only when it contains its Intrinsically Disordered, C-terminal Domain (IDR). The IDR in Rim4 contains 4 out of the 6 PKA consensus sites R R/K X S/T found in the protein and so they mutate these sites to alanine (4A) and glutamic acid (4E) and study their function. Surprisingly these mutations do not impact Rim4 aggregate formation or translation-and thus the authors conclude that PKA must also act through some other factor to regulate Rim4.

I do not find the analysis above compelling for a couple of reasons.

First, it is very likely that the antibody can only recognize a subset of the PKA target sites. For example, the six PKA sites listed in the paper are RRYT, RRFS, RKFS, RKMS and RRKS (in order) -note however I cant see any site at 612 do the authors mean S607. It seems very unlikely that the antibody is going to detect all of these sites equally well-and this does not even account for differences at the plus 1 position. Thus, it could be that the conclusions above (that PKA is not necessary or sufficient to regulate Rim4) are wrong-it is just that the authors need to knock out the other PKA sites to see the effect.

Second there may be PKA dependent phosphorylation at other (possibly non-consensus sites) not considered in the paper (or detected by the PKA phosphosite antibody).

I therefore recommend that the authors (i) map Rim4 phosphorylation in the presence and absence of PKA activity by mass spectrometry and (ii) test the role of the full set of PKA sites they find

Response to referees for manuscript # EMBOJ-2022-113332

Cells couple assembly and function of a disordered translational repressor with nutrient conditions

Diana S.M. Ottoz, Lauren C. Tang, Annie E. Dyatel, Marko Jovanovic, and Luke E. Berchowitz

Our point-by-point responses are in blue.

We would like to thank the referees for taking the time to read our manuscript and for giving us constructive comments and suggestions. We implemented most of the suggested changes and we think that our work improved considerably from your comments.

Referee #1:

Previous results provided evidence that yeast Rim4 is an RNA-binding protein that forms amyloid-like assemblies in meiosis and functions to repress the translation of certain meiotic genes in a manner requiring two of its three RRMs and its intrinsically disordered region (IDR), which is also necessary for assembly formation. These results suggested that translational repression depends on formation of Rim4 assemblies but how assembly formation promotes repression is not understood; nor was it understood how formation of Rim4 assemblies is controlled.

In this report, the authors showed that Rim4 can form aggregates and repress reporter mRNAs harboring the 5'UTRs of two Rim4 target mRNAs when expressed ectopically in mitotic cells. This is significant because it eliminates an essential role for any other meiosis-specific factor at least for these two targets; although a third Rim4 target reporter mRNA was not repressed so that other factors could still be important for certain transcripts.

They confirm the requirement for the Rim4 IDR for assembly and also show that the IDR is sufficient for assembly in mitotic cells. They provide evidence that RNA-binding by Rim4 is highly induced by the starvation conditions that induce sporulation and provide evidence that, unlike the RRMs, the IDR is dispensable for RNA binding; although the PNK assay they employed for RNA binding is rather indirect and does not necessarily reveal binding of authentic Rim4 mRNA targets. In addition, the evidence that the IDR is completely dispensable for RNA binding is somewhat questionable as it appears to be contradicted by the data presented in Supp. Fig.2D. Confirming that the IDR is required for reporter repression, they infer that assembly formation is necessary for translational repression in a manner that goes beyond target mRNA binding per se. However, contrary to statements in the Abstract and Discussion, they do not elucidate the molecular mechanism of translational repression via Rim4 aggregates, which still remains unknown.

We thank the referee for these comments, to which we replied below (Comments 1, 2, and 9).

They attempt to define the nutrient signals that trigger assembly formation and find that replacement of glucose with nonfermentable carbon sources in nitrogen-rich medium is sufficient to trigger assembly formation and reporter repression, whereas starvation for nitrogen permits partial assembly and reporter repression in the presence of glucose. These results are significant in suggesting that the absence of glucose is the nutrient signal that elicits Rim4 assembly and repression.

Experiments using an analog-sensitive allele of TPK1 indicate that inhibiting PKA function is sufficient to trigger Rim4 assemblies in rich glucose medium. This should lead to Rim4-dependent reporter repression as well, but the relevant results were confounded by repression of the reporter mRNA in the absence of Rim4 on inhibition of PKA function, with little or no additional repression conferred on induction of Rim4.
As an alternative approach, they showed that activation of PKA in starved cells by expressing the dominant RAS2Val allele eliminated Rim4 assemblies and seemed to abolish Rim4 repression of the reporter. This experiment (Fig. 3G) was also flawed however because the magnitude of Rim4 repression in the WT control cells was less 2-fold, compared to 5-fold or

more in other experiments. This complication was coupled with unexpected induction of the reporter mRNA by RAS2Val in the absence of Rim4. Thus, while the evidence is strong that PKA impedes Rim4 assembly formation, it is not compelling that PKA also impairs Rim4 translational repression. Other experiments, possibly ones that avoid using the estradiol induced reporter to assay repression, would be required to bolster this key conclusion.

We address these points below, in Comment 3.

They go on to provide evidence, obtained using anti-phospho antibodies that are presumably specific for PKA phosphosites, that the Rim4 IDR is phosphorylated by PKA in rich medium. Unfortunately, however, it was not established that PKA is required for IDR phosphorylation.

We disagree with this conclusion of the referee. We showed that RFP-IDR^{Rim4} is phosphorylated in medium containing glucose and un-phosphorylated in starvation, when PKA is on and off, respectively (Fig. 4B). Moreover, we showed PKA-dependent phosphorylation of full-length Rim4 upon PKA activity manipulation (Fig. EV4A,B). Finally, we observed that the phospho-PKA antibody does not bind to Rim4^{4A} and Rim4^{4E}, where the four PKA consensus sites located in the IDR have been mutated (Fig. EV4C). Therefore, we can deduce that the sites being differentially phosphorylated upon PKA activity manipulation in Fig. EV4A,B are the ones located in the IDR.
Furthermore, our new mass spectrometry data show a PKA-dependent phosphorylation of S470, S525, and S612, three PKA sites located in Rim4 IDR (see Expanded View Table 1).

Moreover, substituting 4 putative PKA sites in the IDR of chromosomal RIM4 with phosphomimetic Glu residues did not impair assembly formation or reporter repression in starved cells in the manner that would have been expected if PKA directly impairs Rim4 assembly by phosphorylating the IDR. Thus, it remains unclear how PKA impairs Rim4 assembly.

We agree with the referee's comments. As described in the updated section "The Rim4 IDR is a direct target of PKA" of the Results, we performed mass spectrometry analysis to identify

possible PKA sites influencing Rim4 assembly. Unfortunately, mutation of those sites did not reveal any convincing phenotype. Therefore, our data do not support an essential role of PKA-dependent Rim4 phosphorylation in assembly. As we wrote in the Discussion, we propose that PKA may prevent Rim4 assembly indirectly, possibly by modulating protein-protein interactions between Rim4 and unknown targets of this kinase.

They do find some evidence that the IDR 4-Ala substitution mutant slightly delays the disappearance of Rim4 protein and assemblies in the return to growth from sporulation/starvation conditions on addition of glucose, and also slightly impedes cell growth in this recovery phase; however, it was not convincing that the growth defect results from the persistence of Rim4 protein/aggregates because the growth delay appears many hours after there is no effect of the Ala mutations on Rim4 protein or assembly abundance.

We appreciate the referee's thoughtful critique of our work, but we disagree with this comment. Upon addition of glucose to sporulation medium, delays in the transition from sporulation to vegetative growth will be visualized several hours later in a growth curve because cells are growing exponentially. That is, since the cell density is very low at 0 hours of return to growth, differences in growth are difficult to visualize early in the curve, but they become more evident hours later, when the cell density is higher.

In summary, it is both convincing and interesting that glucose is the most important nutrient that impedes Rim4 assembly and that PKA function is somehow involved in this. Important drawbacks however are that (i) the mechanism of PKA function in impeding Rim4 assemblies is unclear-it does not seem to involve direct Rim4-IDR phosphorylation-and (ii) the lack of compelling evidence that PKA function also blocks translational repression by Rim4. The evidence that Rim4 RNA-binding function is necessary but not sufficient for translational repression is promising, but there are concerns about the indirect nature of the PNK assay and the data obtained that provides evidence that eliminating the IDR had no effect on RNA binding, and corroborating evidence from RNA-IP is likely required to bolster this point.

We address these comments below:

Major comments:

1 The PNK assay for RNA binding is not specific for Rim4 target mRNAs, nor it seems even for messenger RNA. It would be highly desirable to replace it with an RNA-IP assay (or even better RIP-Seq) to interrogate the effects of the RRM and IDR mutations on Rim4 binding to bone fide target mRNAs and try to confirm that the IDR is completely dispensable for binding such transcripts in starved cells. The large variance of the data for the IDR deletion shown in Fig. 2E underscores a shortcoming of this assay in establishing this key conclusion about the IDR in RNA binding by Rim4.

We agree with the referee that the PNK assay is not specific for detecting interactions between a protein of interest and its target RNAs. However, the unspecific nature of this assay helped us to rule out that the Rim4 IDR binds any nucleic acid. This result is important to understand how Rim4 mechanistically represses translation, as it has been shown that several IDRs are able to interact with RNA (Järvelin et al. 2016).

We are thankful to the referee for suggesting a more specific assay like RNA-IP to monitor RNA-Rim4 interactions. We established this method in the lab and coupled it with qPCR. To understand the role of the Rim4 IDR in RNA binding, we investigated the interactions between Rim4 and the YFP reporter transcript carrying the *CLB3* 5′UTR. To test the specificity of the assay, we chose to monitor the *CUP1-1* transcript, which is not targeted by Rim4 and, together with *RIM4*-V5, is highly induced by copper in our experimental setup (Fig. EV1D). Our RNA-IP results showed that the IDR is necessary for efficient binding of a target mRNA in starvation (updated Fig. 2D). However, the role of the IDR is indirect because it does not bind RNA itself. Therefore, we conclude that Rim4 assembly, mediated by its IDR, is necessary for function because it is required for efficient binding of its mRNA targets. We feel that the results we obtained with our reporter YFP are sufficient to give us a clear idea of the role of the Rim4 IDR in RNA binding, therefore we think that RIP-seq is not necessary to further support our conclusions.

In our hands, the RNA-IP/qPCR method is noisy. This is reflected by the high variance of the data, and in the statistical analysis, which did not reveal significant differences in several sample comparisons (we think the lack of statistical significance also stems from the need to use a non-parametric test, as the data are not normally distributed). Nevertheless, we are very confident of our RNA-IP results and think that they helped to clarify important aspects of our work. Despite the high variance, the RNA-binding behavior of our Rim4 mutants or of wild-type Rim4 in different conditions shows very clear and interpretable trends. Moreover, the binding behavior of wild-type Rim4 was reproducible across the three sets of independent RNA-IP experiments we conducted (Fig. 2D,F and Fig. EV2B), further supporting our findings (note that we could not merge the biological replicates of these experiments for wild-type Rim4 because of experiment-specific adjustments we had to carry out to account for differences of pulled-down Rim4, see Methods for the details).

2 The data in Fig. S2D on the IDR deletion variant seems to indicate a clear reduction in binding in the phosphoimage that cannot be explained by the anti-V5 (Rim4) immunoblot. These results would have to represent rather extreme outliers among replicates to be reconciled with the mean RNA/protein ratios shown in Fig. 2E for this variant. Also, statistical analysis is lacking for the results in panel E, making it unclear if the 3-rrm mutant truly has a reduced RNA/protein ratio.

We thank the referee for pointing out this apparent discrepancy. Data shown in old Figure 2D represented a 1:10 dilution of the undiluted samples shown in old Figure S2D. Unfortunately, we did not clarify this point in the Figure legend. We now added a new replicate of the PNK assay where we show a 1:10 dilution of all samples (both UV crosslinked and un-crosslinked, see Fig. EV1C). However, we decided to leave out the PNK assay quantifications from the manuscript, as we further analyzed the ability of the Rim4 mutants to bind RNA with RNA-IP, which is more specific (Fig. 2D). With this method, we confirmed that neither RFP-IDR^{Rim4} nor the 3-rrm mutant are able to bind the YFP mRNA. Moreover, RNA-IP revealed that Rim4ΔIDR does not bind the YFP mRNA efficiently. This is in contrast with the PNK assay results, which showed that Rim4ΔIDR binds nucleic acids. This discrepancy may depend either on the

unspecific nature of the PNK assay (that is, as the referee suggested, that we detected, with the PNK assay, interactions between Rim4ΔIDR and other RNA species) or on the stringency of the RNA-IP assay (For this last point, it is important to note that we did not include a crosslinking step with UV in our RNA-IP procedure, while samples were crosslinked for the PNK assay). Therefore, our RNA-IP suggests that, if Rim4ΔIDR binds target transcripts, it does it with lower affinity than full-length, wild-type Rim4, corroborating the idea that assembly promotes efficient RNA binding. We tried to include a UV-crosslinking step in our RNA-IP to explore this possibility, but we encountered RNA or protein degradation. The IP protocols we used for the PNK and RNA-IP assays differed in several aspects. For the PNK assay, we treated the cell pellets with TCA and denatured the whole cell extracts with SDS/boiling before performing the immunopurification. For the RNA-IP assay, we did not treat the cells with TCA, and we performed immunopurification of Rim4 under native conditions. In our hands, we encountered RNA degradation during extraction from TCA-treated denatured whole-cell extracts. Furthermore, when we tried native IP on UV-crosslinked samples, we encountered Rim4 degradation.

3 Additional experiments are required to show that inhibition of PKA in the tpk1-as strain or activation of PKA in the RAS2val strain alters translation of a Rim4 reporter in the predicted manner, overcoming the confounding effects of PKA function on the estradiol-induced reporter and the poor repression ratio for the WT cells in Fig. 3G.

The referee rightfully pointed out that in old Fig. 3G the magnitude of Rim4 repression in the wild-type control is less than in other experiments presented in the manuscript. The reason for this is the experimental setup, and we apologize for not explaining it properly. Here, we wanted to check the ability of Rim4 to repress translation in a strain overexpressing *RAS2*^{G19V} in starvation. In these strains, both the YFP reporter and *RAS2*^{G19V} are controlled by beta-estradiol and induced *before* transferring the cells to starvation. This means that cells accumulated YFP *before* starvation, that is, *before* Rim4 assembled and started repressing translation. In the other experiments described in the manuscript YFP is always induced *after* transferring the cells to starvation, concomitantly with *RIM4*-V5 expression induction. To be able to interpret the *RAS2*^{G19V} results correctly, we induced the wild-type control strains with

the same scheme as we did for the RAS strains. Therefore, in this experiment, the magnitude of repression in the wild-type strain is lower than in the other experiments described in the manuscript. To make sure that this point is clear to the readers, we explain the induction scheme in the main text and provide explanatory figures (Fig. EV2D and Appendix Fig. S2).

We followed the suggestion of the referee and set up a complementary assay with a strain expressing *RAS2*^{G19V} from the *CUP1-1* promoter (the same that controls *RIM4*-V5). The data nicely mirrored those where *RAS2*^{G19V} was induced with beta-estradiol, and we could confirm that, when PKA is ectopically activated in starvation, Rim4 is not able to form assemblies nor to repress the reporter gene (Fig. EV2E,F and Appendix Fig. S4E,F).

We also decided to perform RNA-IP in the *tpk1-as* strain to check the possibility that the Rim4 assemblies are not able to repress translation because they are not able to bind the reporter transcript upon inactivation of PKA in glucose-containing medium. This hypothesis was disproved by the RNA-IP data, as Rim4 is able to bind the reporter mRNA in this context (Fig. EV2B). In the Discussion, we mention the possibility that assembly-dependent RNA binding might not be sufficient for translational repression. However, please note that ectopic PKA inactivation causes growth arrest and eventually cell death, complicating the interpretation of these results.

4 Additional text is required for the interpretation of the gels quantifying Rim4 assemblies. Fig. 3A gives the impression that the amount of Rim4 assemblies in N-starved cells grown with glucose are actually higher than those in rich medium with glycerol or acetate, but this is unlikely to be true considering that the overall amount of Rim4 on conventional SDS-PAGE doesn't vary for these conditions (Fig. S3A). To get a better estimate of the amounts of assemblies, they could quantify the proportion of total signal found at the top of the gel in the assembly bracket from replicate experiments. Presumably this would help overcome the high variability of cumulative signal throughout the lane, which can vary greatly but is expected to be nearly constant.

We thank the referee for this comment. SDD-AGE is a qualitative assay to analyze the ability of a protein to form SDS-resistant aggregates and we do not attempt to interpret the data in a quantitative manner. This assay allows to distinguish assemblies, running at molecular weight in the range of mega Daltons, from monomers or non-assembled proteins, running at their actual molecular weight in the range of kilo Daltons. Note that the resolution power of SDD-AGE is limited as we use agarose gels and the aggregates' size can be very large. Therefore, in Fig. 3A we describe the presence or absence of monomers and aggregates, without commenting on the signal intensities.

There are caveats that prevent the SDD-AGE assay from being quantitative. First, the protein extract preparation: this requires two subsequent clarification steps of native lysate that may cause differential pelleting of the assemblies based on their structure and/or size. For example, it could be that the average size of the assemblies formed in the different growth conditions tested in Fig. 3A is different, resulting in different signal intensities. Second, the transfer: entities with higher molecular weight are transferred less efficiently to the membrane, while monomers, which are smaller, are transferred more efficiently. For this reason, visualization of monomers allows us the be confident that Rim4 exists also as a monomer in the conditions tested.

5 Fig. 2B: the data for the 3-rrm mutant here is unsatisfying as the total signal is unexpectedly very low; the same is true for the SPO+glucose condition in Fig. 3A.

We substituted panels in old Fig. 2B and old SUPP. Fig. 2B with a replicate whose signal is clearer (now in Fig. 2B and Fig. EV1A).
Please also consider our comments above regarding the differences in signal intensities in SDD-AGE gels.

6 Line 271: the claim that Rim4IDR(4A) protein levels were higher compared to wild type or Rim4IDR(4E) during sporulation is not supported by quantification of Western data from replicate experiments of the kind shown in Fig. 5A. If it is convincing merely from visual

inspection of replicates, this should be stated and the replicates cited in the Source data, and the magnitude of the increase should be quantified from the replicates.

We thank the referee for this point. We made a mistake in the sentence by stating that Rim4^{4A} was more abundant than wild-type and Rim4^{4E} during sporulation. Indeed, we don't think the levels of Rim4 are clearly different in meiosis. Instead, they are different in return to growth. Therefore, we changed the sentence.

7 Line 293: the conclusion here is too strong as there is no direct evidence that PKA phosphorylates the Rim4 residues S525 and S612 implicated as sites of 14-3-3 protein binding in their previous paper, only a correlation between phosphorylation and PKA activity.

We thank the referee for this comment. We agree that our work shows a correlation between PKA activity and Rim4 phosphorylation during meiosis, and that we cannot exclude the possibility that these phosphorylation events are indirect. Therefore, we changed the wording of the conclusive sentence of the "Rim4 phosphorylation by PKA contributes to Rim4 assembly clearance" section to underline that "PKA-dependent phosphorylation" events are involved in Rim4 assembly clearance during return to growth and meiotic progression.
However, we think that the experiments we described overall strongly support the idea that Rim4 is phosphorylated in a PKA-dependent fashion. Moreover, our recent mass spectrometry results conducted in mitotic cells demonstrated that S525 and S612, which are located in PKA consensus sequences, are phosphorylated in a PKA-dependent fashion.

8 The writing suffers overall in lacking explicit information that would assist the reading in grasping their data and interpretations of results. There is insufficient citing of particular results in the text, especially for data shown in the Supplementary figures which are merely mentioned in passing. One gets the impression that the manuscript was prepared for publication in a journal with a much more restrictive character limit, preventing a "user-friendly" description of details of assays and key results obtained.

We thank the referee for pointing this out. This revision includes more detailed descriptions of our assays and results.

9 Lines 31-32: This claim in the Abstract is overstated. The mechanism for how PKA impedes Rim4 assembly is still unknown, as is the mechanism for how Rim4 assembly formation supports translational repression.

We thank the referee for this comment. We agree that many aspects of how Rim4 assembly represses translation are still unknown and need to be explored further. Therefore, we softened the wording of our statements in both Abstract and Discussion.
Nevertheless, the data we presented here contribute to elucidate several aspects of how Rim4 assemblies repress translation. First, our mitotic assay helps in ruling out possible meiotic factors involved in the repression mechanism. Second, we identified the growth condition requirements for Rim4 function. Third, we show that assembly is necessary for function. Fourth, we show that both RRMs and IDR are necessary for function, as the RRMs are the sole domains that can bind RNA and the IDR allows for efficient RRM function through assembly formation. Finally, we expanded our analysis of how Rim4 assemblies repress translation of select transcripts. We added a new set of data in Fig. 2E-G, Fig. EV1E,F, and Appendix Fig. S3B,C and a new section entitled “The location of the Rim4 binding sites within a transcript is important for translational regulation”, which show that the position of the Rim4 binding sites within the target RNA is fundamental for translational outcome. These results support a model where Rim4 assemblies form a polarized RNA-protein structure architecture with the target RNA that make the latter inaccessible to the ribosomes.

Other comments:

10 Should stipulate that "starvation conditions" always correspond to sporulation conditions

We thank the referee for this comment. We now added a sentence in the first Results section ("Rim4 assembly and function can be transferred to starved mitotic cells") where we pointed out that, unless specified otherwise, we starved the cells with sporulation medium.

11 The authors should note the confounding issue that the reporter mRNA is not induced in nitrogen starvation medium or SPO medium supplemented with Glucose (Fig. 3B). Is this understood?

In our experimental setup, we induced the cells with β-estradiol just one hour after transferring them from a rich medium (YPD) to starving conditions. We think that when the cells are hit by the inducer, they are still adapting to these challenging growth conditions. The response triggered during this media transitions depends on the quality and quantity of nutrients available. Therefore, we think it is reasonable that cells adapting to media combining nitrogen limitation with or without a fermentable carbon source display different abilities to induce the expression of the reporter gene.

12 -line 211: At odds with this statement, the induction of Rim4 does not appear inefficient in Fig. S3D.

We thank the referee for this comment. After inspection of different exposures of our western blots we agree with the referee and have amended the text.

13 Line 305: this is not true, as the role of assembly in repression is still a mystery

We have addressed this comment in our responses to Comments 1,2, and 9.

Referee #2:

This work from Ottoz et al provides interesting and important new insights into the way that yeast controls the movement of Rim4 into amyloid-like aggregates, and the concomitant Rim4 dependent repression of translation, during the transition into meiosis. Specifically, the authors show that the PKA kinase phosphorylates Rim4 in the presence of glucose and this blocks the assembly of Rim4 into insoluble aggregates. Then once cells inter starvation conditions, PKA is inhibited, driving the Rim4 dependent repression of translation.

The experiments presented are very well done and convincing up until the authors start to pick apart the role of individual phosphorylation sites in Fig. 4. Specifically, once the authors show that PKA controls the movement of Rim4 into aggregates and the Rim4 dependent repression of translation, they set out to determine which if any of the PKA sites on Rim4 drive these events. To do this they follow the phosphorylation of Rim4 in different conditions using an anti-PKA phosphosite antibody. They find that Rim4 reacts with this antibody in glucose, but only when it contains its Intrinsically Disordered, C-terminal Domain (IDR). The IDR in Rim4 contains 4 out of the 6 PKA consensus sites R R/K X S/T found in the protein and so they mutate these sites to alanine (4A) and glutamic acid (4E) and study their function. Surprisingly these mutations do not impact Rim4 aggregate formation or translation-and thus the authors conclude that PKA must also act through some other factor to regulate Rim4.

I do not find the analysis above compelling for a couple of reasons.
First, it is very likely that the antibody can only recognize a subset of the PKA target sites. For example, the six PKA sites listed in the paper are RRYT, RRFS, RKFS, RKMS and RRKS (in order) -note however I cant see any site at 612 do the authors mean S607. It seems very unlikely that the antibody is going to detect all of these sites equally well-and this does not even account for differences at the plus 1 position. Thus, it could be that the conclusions above (that PKA is not necessary or sufficient to regulate Rim4) are wrong-it is just that the authors need to knock out the other PKA sites to see the effect.

We agree with the referee that the anti-phospho PKA site antibody may not recognize all six phosphorylated PKA sites of Rim4 with the same efficiency. Therefore, we proceeded in substituting all six consensus PKA sites of Rim4 with alanine or glutamic acid residues (here listed: RRYT, RRFS, RKFS, RKMS, RRKS, and RRSS. Note that in the SK1 yeast background we use Rim4 contains an insertion at position +1766 from the start codon (ACAACAACAACAAAA) compared to S288C. Therefore, in SK1 the sixth PKA site is at position 612 instead of 607). Unfortunately, we did not observe any change in assembly or function of Rim4 (see Fig. 4D,E and Appendix Fig. S5B).

Second there may be PKA dependent phosphorylation at other (possibly non-consensus sites) not considered in the paper (or detected by the PKA phosphosite antibody).
I therefore recommend that the authors (i) map Rim4 phosphorylation in the presence and absence of PKA activity by mass spectrometry and (ii) test the role of the full set of PKA sites they find

We thank the referee for proposing these experiments. We performed mass spectrometry on Rim4-V5 immunopurified from cells grown in YPD (where PKA is active) and SPO (where PKA is inactive). To confirm that the phosphorylation events we observed in these two conditions were PKA-dependent, we also performed mass spectrometry on strains where PKA was either ectopically inactivated or activated. For this mass spectrometry experiment we used a special protein digestion protocol. Conventional trypsin digestion would not digest Rim4 into fragments that can be easily detected by mass spectrometry and that can be confidently mapped back to the sequence, because a large portion of the Rim4 IDR is devoid of lysine and arginine residues. Therefore, to circumvent this problem and achieve the greatest coverage of Rim4, we split each sample in two halves. One half was digested with trypsin and glu-c and the other with chymotrypsin (Appendix Fig. S6).

The data we obtained allowed us to conclude that there is a PKA-dependent phosphorylation of the PKA sites located in the Rim4 IDR (S470, S525, and S612, see Expanded View Table 1). We also detected some phosphorylation signal for S367, however, the dataset for that site was incomplete, therefore, we do not feel that we can conclude much about S367. The mass

spectrometry experiment identified residues surrounding the S470 and S525 PKA sites that may also be targeted in a PKA-dependent fashion (Fig. EV4D and Expanded View Table 1). Moreover, we identified two sites in the N-terminus of Rim4 (S10 and S16) whose phosphorylation profile anticorrelated with PKA activity, suggesting that PKA may impede Rim4 assembly indirectly by preventing some phosphorylation events. We were intrigued by these findings and constructed and analyzed mutants where we mutated all the phospho-sites identified by mass spectrometry displaying both a positive and negative relation with PKA activity (Fig. EV4E). To our disappointment, these mutations had no or very little effect on Rim4 assembly and its ability to repress translation (see Fig. 4D,E). Therefore, we conclude that PKA-dependent phosphorylation of Rim4 is not sufficient or necessary to control its assembly and function. As we wrote in the Discussion, we propose that PKA may prevent Rim4 assembly indirectly, possibly by modulating protein-protein interactions with unknown targets of this kinase.

We deposited the mass spectrometry dataset on MassIVE:
https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp?redirect=auth
temporary login credentials:
username: MSV000092632_reviewer
password: a
ftp address:
ftp://massive.ucsd.edu/MSV000092632/

References

Järvelin AI, Noerenberg M, Davis I, Castello A. 2016. The new (dis)order in RNA regulation. *Cell Commun Signal* **14**: 9.

Dear Luke,

Congratulations on a great revision! Overall, the referees have been positive. However, referee 1 has two (non-experimental) suggestions that we ask you to address in a revised version.

When you submit your revised version, please also take care of the following editorial items and add this also to your point-by-point response:

1. The grant number R35 GM128802 is missing in the manuscript file, please add.

2. Please provide up to five keywords, which may or may not appear in the title, should be given in alphabetical order, below the abstract, each separated by a slash (/).

3. Please review our new policy on conflict of interests on the EMBO author guide website and update the title of this section to: Disclosure and competing interests statement.

4. Please remove the author contribution section from the main manuscript file.

5. For the author checklist, please correct the typo in the general table.

6. Please rename Table EV1 to Dataset EV1 (with the corresponding callout in the manuscript updated) and upload this individually (not zipped).

7. Please remove the EV1 legend from the manuscript and include it as a separate sheet in the Excel file.

8. Please add page numbers to the table of contents in the appendix file.

9. We require the publication of source data, particularly for electrophoretic gels and blots and graphs, with the aim of making primary data more accessible and transparent to the reader. It would be great if you could provide me with a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure or for graphs, an Excel spreadsheet with the original data used to generate the graphs. The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight marker; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files. If you require assistance with this, please contact h.sonntag@source-data.org.

10. We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

11. We also need a summary figure for the synopsis. The size should be 550 wide by 200-440 high (pixels). You can also use something from the figures if that is easier.

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

Kind regards,

Kelly

Kelly M Anderson, PhD
Editor, The EMBO Journal
k.anderson@embojournal.org

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/

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Referee #1:

The manuscript has been improved by using RNA-IP assays to monitor specific Rim4 binding to the YFP reporter in a manner dependent on the CLB3 5'UTR, providing stronger evidence that the IDR is important for RNA binding as well as for formation of Rim4 assemblies and translational repression. They also provided much stronger evidence in Fig. EV2D-F that ectopic activation of PKA by RAS2Val impairs translational repression of the YFP reporter by Rim4 in starved cells. Although they have demonstrated convincingly that PKA phosphorylates Rim4, and their evidence is strong that PKA function is required to impede Rim4 assembly in rich medium and that inappropriate PKA activation in starvation conditions prevents formation of Rim4 assemblies and translational repression by Rim4, the critical PKA substrates involved in controlling Rim4 assembly and function remain unknown, as Ala and Glu substitutions of the PKA phosphosites in Rim4 have no impact on steady-state assembly of Rim4 aggregates or translational repression. Nevertheless, their results do show that blocking phosphorylation of Rim4 phosphosites with Ala substitutions delays the repression of Rim4 expression and the loss of Rim4 assemblies when meiosis is interrupted by returning to rich medium. Thus, it seems that direct IDR phosphorylation by PKA plays at least some role in controlling translational repression by Rim4 in sporulating cells. The manuscript is also improved by the addition of new data indicating that the binding site for Rim4 cannot function in translational repression if it is located in the 3'UTR vs. 5'UTR, suggesting that the mechanism of repression involves preventing ribosome attachment or scanning of the 5'UTR to select the start codon, as opposed to simply sequestering the mRNA in a Rim4 aggregate.

-I suggest that the authors replace the existing Fig. 3G-H with the stronger results shown in Fig. EV2E-F.

-I suggest that the authors refrain from concluding both in RESULTS and DISCUSSION that the assembly and translational repression activities of Rim4 were uncoupled on inhibition of PKA function in the experiment of Fig. 3F because the unexpected repression of the YFP reporter on inhibition of PKA in cells lacking Rim4 confounds their ability to determine whether or not translational repression by Rim4 is intact.

Referee #2:

The authors dramatically improved their analysis of Rim4 phosphorylation and its impact on Rim4 aggregation and the translation of Rim4 target mRNAs (as requested). Unfortunately, despite the authors best efforts, mutating in the PKA dependent phosphorylation sites on Rim4 did not block PKA dependent Rim4 aggregation. This means that the mechanism underlying the PKA dependent regulation of Rim4 remains a mystery and this lowers the impact of the paper. Despite this, the work is technically sound and represents a significant step forward in understanding Rim4 regulation/ function and the role of stress and starvation signaling in regulating protein aggregation and cell function.

Our point-by-point responses to the referees' comments are in blue.

Referee #1:

The manuscript has been improved by using RNA-IP assays to monitor specific Rim4 binding to the YFP reporter in a manner dependent on the CLB3 5'UTR, providing stronger evidence that the IDR is important for RNA binding as well as for formation of Rim4 assemblies and translational repression. They also provided much stronger evidence in Fig. EV2D-F that ectopic activation of PKA by RAS2Val impairs translational repression of the YFP reporter by Rim4 in starved cells. Although they have demonstrated convincingly that PKA phosphorylates Rim4, and their evidence is strong that PKA function is required to impede Rim4 assembly in rich medium and that inappropriate PKA activation in starvation conditions prevents formation of Rim4 assemblies and translational repression by Rim4, the critical PKA substrates involved in

controlling Rim4 assembly and function remain unknown, as Ala and Glu substitutions of the PKA phosphosites in Rim4 have no impact on steady-state assembly of Rim4 aggregates or translational repression. Nevertheless, their results do show that blocking phosphorylation of Rim4 phosphosites with Ala substitutions delays the repression of Rim4 expression and the loss of Rim4 assemblies when meiosis is interrupted by returning to rich medium. Thus, it seems that direct IDR phosphorylation by PKA plays at least some role in controlling translational repression by Rim4 in sporulating cells. The manuscript is also improved by the addition of new data indicating that the binding site for Rim4 cannot function in translational repression if it is located in the 3'UTR vs. 5'UTR, suggesting that the mechanism of repression involves preventing ribosome attachment or scanning of the 5'UTR to select the start codon, as opposed to simply sequestering the mRNA in a Rim4 aggregate.

We thank the referee for the positive comments.

-I suggest that the authors replace the existing Fig. 3G-H with the stronger results shown in Fig. EV2E-F.

We thank the referee for this suggestion. We exchanged panels 3G and 3H with EV2E and EV2F, respectively.

-I suggest that the authors refrain from concluding both in RESULTS and DISCUSSION that the assembly and translational repression activities of Rim4 were uncoupled on inhibition of PKA function in the experiment of Fig. 3F because the unexpected repression of the YFP reporter on inhibition of PKA in cells lacking Rim4 confounds their ability to determine whether or not translational repression by Rim4 is intact.

We thank the referee for this comment; however, we do not agree with removing this conclusion completely from the manuscript, as our data do not exclude the possibility

that Rim4 assembly and function may be uncoupled in certain conditions. To make it clear that this is a speculation from our side, we decided to remove this conclusion from the Results section. In the Discussion, we reorganized the sentence to make clear the speculative nature of our statement.

Referee #2:

The authors dramatically improved their analysis of Rim4 phosphorylation and its impact on Rim4 aggregation and the translation of Rim4 target mRNAs (as requested). Unfortunately, despite the authors best efforts, mutating in the PKA dependent phosphorylation sites on Rim4 did not block PKA dependent Rim4 aggregation. This means that the mechanism underlying the PKA dependent regulation of Rim4 remains a mystery and this lowers the impact of the paper. Despite this, the work is technically sound and represents a significant step forward in understanding Rim4 regulation/ function and the role of stress and starvation signaling in regulating protein aggregation and cell function.

We thank the referee for their comments and for reviewing our work.

2nd Revision - Editorial Decision **25th Sep 2023**

Dear Luke,

Congratulations on an excellent manuscript, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal. Thank you for your comprehensive response to the referee concerns and for providing detailed source data. It has been a pleasure to work with you to get this to the acceptance stage.

I will begin the final checks on your manuscript before submitting to the publisher next week. Once at the publisher, it will take about 3 weeks for you manuscript to be published online. As a reminder, the entire review process, including referee concerns and your point-by-point response, will be available to readers.

I will be in touch throughout the final editorial process until publication. In the meantime, I hope you find time to celebrate!

Kind regards,

Kelly

Kelly M Anderson, PhD
Editor, The EMBO Journal
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The data shown in figures should satisfy the following conditions:

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- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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:
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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.

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For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Appendix Table S5

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Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

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Microbes: provide species and strain, unique accession number if available, and source.	Yes	Appendix Table S2

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Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

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Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods

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Studies involving **human participants**: 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	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
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Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	