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The GCN2/eIF2αK stress kinase regulates PP1 to ensure mitotic fidelity

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Dear Dr. Grallert,

Thank you for the submission of your research manuscript to EMBO reports. It has now been evaluated by three expert referees, whose detailed reports are appended below.

As you will see, the referees acknowledge that the findings are novel, significant and interesting, and their reports are broadly favorable. However, they also raise a number of concerns with respect to some of the experimental results, which should be addressed to further substantiate the conclusions of the study. Furthermore, they all provide detailed suggestions for the improvement of the study and the manuscript.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript. If you have any questions or comments, we can also discuss the revisions in a video chat, if you like.

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 (January 9th). Please discuss with me the revision progress ahead of this time if you require more time to complete the revisions.

*****
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7) Please note that a Data Availability section at the end of Materials and Methods is now mandatory. In case you have no data that requires deposition in a public database, please state so instead of refereeing to the database.
See also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

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The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
- If the data are obtained from n {less than or equal to} 2, use scatter plots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

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- Please also include scale bars in all microscopy images.

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Yours sincerely,

Ioannis Papaioannou, PhD
Editor
EMBO reports

Referee #1:

The manuscript by Stonyte and colleagues reports novel and =potentially biologically impactful results, that ascribe a novel role for the amino acid sensing cytoplasmic kinase GCN2. The data supports a role for GCN2 in cell cycle progression through mitosis, which surprisingly is distinct form its major role as a sensor for amino acid availability and regulator of translational control of gene expression. The paper is well written, and the data is, for them most part clear and supports the main conclusions of the paper. The experimental design is robust with multiple biological and technical replicates and frequent use of rescue experiments using wild-type and mutant constructs to demonstrate biological specificity. However, there are some concerns with respect to some of the experimental results, especially with those pertaining to the biological consequence of GCN2 inhibition in determining cell fate.

Major issues:

1. An important question that arises from these results, is why there is a lack of any overt developmental phenotype in GCN2 knockout animals. GCN2-/- mice are viable, fertile and do not present any developmental abnormalities in utero or after birth (they do show some hepatocellular effects under specific diets). Given the important role in mitotic entry and progression in unstressed conditions shown here, one would expect significant defects in organ formation in rapidly growing tissues. The authors need to address this paradox.
2. The hTERT-expressing RPE1 cells, though untransformed, are not "normal". The authors should test the effects of GCN2 inhibition (preferably with GCN2i) in human normal diploid fibroblast or epithelial cells.
3. The immunoblot in Fig. 3A shows highly variable levels of total GCN2 which confound the results. Have the authors tried knocking GCN2 out completely using CRISPR and then transfecting with the different CGN2 constructs? That might achieve a more equal level of expression of total GCN2. Also, the legend indicating a "horizontal line" in the blot should be corrected to a "vertical line".
4. The results in Fig 5 are not convincing, at least as explained in the text. It is stated that GCN2 inhibition changes the localization of AURA and other centrosome proteins form a localized to a diffuse phenotype. Yet, in Fig. 5A it is shown that in the second set of siGCN2 KO cells, AURA remains highly localized. Same goes for the results in Fig. 5B for ChTOG.
5. The immunoblot in Fig. 7D lacks any background. It needs to be reproduced with background shown, as well as quantitation of normalized band densities.
6. The results in Fig.8 are among the most biologically meaningful results of this work; yet, they are far from convincing. First, in terms of quantitation of the number of cells with micronuclei (panel E), there is no statistical comparison between the different groups, especially between the combined Aurora A and GCN2 inhibitors. Does the combination produce significantly higher number of cells with micronuclei than either inhibitor alone? More critically, there is no such comparison in panel I, which shows the impact on cell survival. This is an important point, since the authors emphasize the possibility of using this approach (synthetic lethality) in pathological conditions of GCN2 overactivation (e.g., tumors). Moreover, more robust apoptotic/survival assays need to be employed here to demonstrate proof-of-concept. Staining for Cleaved Caspase-3 and clonogenic survival following treatments with either inhibitor or combined treatments are warranted.

Minor points.
1. Fig. 1A needs a legend to explain what each different row of images represents (like in Fig. 1F below).
2. The legend in Fig. 1D is rather confusing. Recommend changing "GCN2wtSiCtr" to "siCTR-GCN2wt". Same for GCN2wtsiGSN2.

Referee #2:

1. Does this manuscript report a single key finding? YES

2. Is the reported work of significance (YES), or does it describe a confirmatory finding or one that has already been documented using other methods or in other organisms etc (NO)? YES

3. Is it of general interest to the molecular biology community? YES

Suggests new substrates and pathway interactions in highly conserved cell processes which may impact on cancer therapeutic development.

4. Is the single major finding robustly documented using independent lines of experimental evidence (YES), or is it really just a preliminary report requiring significant further data to become convincing, and thus more suited to a longer¬format article (NO)?

YES - with reservations (see below)

Stonyte et al. show a novel finding, namely that GCN2 locates to the nucleus during mitosis, and that GCN2 activity is necessary for timely and correct mitosis. The strongest aspects of the manuscript are the initial observation of mitotic deregulation upon GCN2 silencing, GCN2 co-precipitation with PP1 and phosphorylation of PP1. However, there are significant shortcomings throughout the manuscript with a lack of care over typos, figure errors, controls etc. The manuscript could strongly benefit from a re-examination and correction from the authors. Having said that however, the experiments have been conducted rigorously, and the conclusions are compelling. The finding that inhibition of GCN2 may impact cell cycle progression, and this might be compounded in cancerous cells, is likely to be of interest to a broad readership.

Major Comments

A1 In general, the silencing of GCN2 through siRNA seems to be incomplete. the supplementary data figure (EV1) shows that there is a very poor silencing of GCN2, as there remains significant amount of GCN2 in their HeLa cells post-silencing (~70% of wt). Could the authors comment on the apparent disconnect between their western blot data (Figure 1C) and the data presented in EV1.

A2 Supplementary Videos are insufficiently described and labelled. On Line 99, the authors refer to supplementary videos in an imprecise manner which limits their utility. If the videos are to be included in the manuscript, then the authors should annotate them sufficiently. For example, there are three videos labelled A0 G0, which is extremely ambiguous.

A3 The authors chose to activate GCN2 using UV irradiation of cells. This is problematic on several levels. Firstly, although GCN2 has been shown to be activated through UV exposure, it is perhaps the least 'canonical' route to GCN2 activation, and the mechanism is very much poorly understood. Secondly, UV irradiation is almost certainly going to cause a DNA damage response, and this may account for the perturbation of mitosis. Thirdly, although the authors make use of Halofuginone in EV3 (the standard compound for GCN2 activation), this is not the method of choice in Figure 3, which leads to a disconnect in the data.

A4 There has been suggested to be significant overlap between GCN2 and PERK, and a functional redundancy in the signalling pathway when either is inhibited. Have the authors explored whether PERK can account for the delay, as opposed to the arrest, or mitosis?

A5 Figure EV3 B and C, Figure EV4A, EV6A, 6A, 6F, - Loading Controls are missing for blots. In general, the quality of the blots is sufficient, but these controls are necessary. Would the authors be able to provide loading controls for all blots?

A6 Have the authors assessed whether GCN2AAXA is active or correctly folded? Does treatment with Halofuginone still elicit an ATF4 phosphorylation/autophosphorylation response?

A7 The authors use an extremely narrow range of GCN2i - between 1 and 3 micromolar. Do they have data supporting this range of concentrations, showing that at 3 micromolar that GCN2 is entirely inhibited and no longer phosphorylates eIF2alpha on exposure to halofuginone?

A8 In most anaphase timing experiments; the x axis extends to 150 minutes. However, in Figures 2C, 7A and 7B this is not the case. This extends to a wider point - how was significant deviation for anaphase timing calculated? Is the significance calculated at the 150-minute timepoint, or the midpoint of the curve?

Minor Points:

As previously mentioned, the manuscript has many typographical errors. Here is a selection.

B1 Line23: The statement requires adequate citation.
B2 Line28: The statement requires citation.
B3 Line72: "few phosphatases stand for the removal of these phosphates". I am not certain as to the meaning of this phrase - consider rewording
B4 Line80: Capitalisation of "NOT" is unnecessary.
B5 Figure 1D. The outline of siGCN2 significance bands is a different colour to the main line, which is confusing.
B6 Line 125 Typo "rolw"
B7 None of the cell sorting data figures have sufficient details for axes.
B8 Figure EV3 C - Please specify the antibody used for GCN2-P (i.e. which site is being probed)
B9 Line 125-127 - "downstream consequences" rephrase as ambiguous
B10 Figure 3A - "The vertical line on the GCN2 blot indicates that the bands
shown for the non-transduced cell line ("wt") are a longer exposure of the same blot." If I follow correctly, these are two expsoures, both in wt cells. But the loading controls remain the same? How is that possible?
B11 Line 152 Typo "GNC2"
B12 Clarity is required for when the authors are silencing the endogenous GCN2 (siGCN2) or using the silencing resistant GCN2. The use of GCN2wt to denote the use of a silencing resistant GCN2 expression is very confusing.
B13 Figure 4A typo - "Bovin"
B14 The term in vitro is inconsistently italicised throughout.
B15 Figure 7C - labels on x axis are poorly formatted.
B16 Figure 7 Figure legend. Typo "GNC2"
B17 Figure EV6C - x axis labels oddly spaced
B18 Figure EV7B/D Either 7,5 or 7.5 - not both.
B19 References are highly variable in format.

Referee #3:

In this manuscript, Stonyte et al. investigated a potential function of GCN2 that is independent of its canonical role in eIF2α phosphorylation and translation regulation, although GCN2 has been generally regarded as a protein translation regulator based on its kinase activity toward eIF2α. In this manuscript, the authors show that GCN2 can localize at centrosomes during mitosis, and likely this pool of GCN2 is involved in the regulation of mitosis by phosphorylating two substrates PP1α and PP1γ. Indeed, both PP1α and PP1γ can be phosphorylated by GCN2 in in vitro kinase assay, and their phosphorylation status is also altered in vivo when GCN2 is depleted. They also show that pharmacological GCN2 inhibition leads to mitotic errors, which renders synergistic effect with Aurora A inhibition. These findings shed light on the unappreciated roles of GCN2 stress kinase in mitotic fidelity. In principle, dissecting if/how eIF2aK4-GCN2 stress kinase regulates PP1 would be of interest, however, this study only establishes the connection but does not conclusively demonstrate this. The authors should address the following points prior to publication.

Major points:
1. The title of "Figure 1. GCN2 depletion leads to a delay through mitosis." does not represent the whole conclusion of the data shown in this figure, because the data also showed that GCN2 is localized at centrosomes during mitosis, which is a novel observation.

2. Figure 1B and 1D: How the time point 0 min was defined? The authors did not explain how this was done in either the main text and the legend for the figure, or in the Methods section. What criteria were used, for example, the markers for prophase, metaphase, or anaphase? I guess these experiments were performed with n = 6 or 5 cells for each sample, namely the sample size, rather than n = 6 or 5 independent experiments respectively. If this is true, then I would think that the sample size was pretty small. In addition, I strongly suggest the authors reselect the color for each chart/sample, because the current colors used are difficult to be distinguished by readers. The same issue is applied to Figure 3B and 4C.

3. Figure EV1 (should be Figure S1): It seems the depletion of GCN2 at centrosomes is rather incomplete based on the representative images authors provided, but in Figure 1C, the GCN2 depletion seems rather complete.

4. I wonder how specific the anti-GCN2 antibodies are? To definitely show or confirm the localization of GCN2 at centrosomes, I would suggest the authors to employ CRSPR-Cas9 to knock-in GCN2-GFP, thus avoid the specificity issue of immunofluorescence.

5. In Figure S1B, the scattered dots shown presumably represent the relative fluorescence intensities of individual GCN2 signals in each cell measured, but the numbers of measured GCN2 signals (based on depicted dots in the graph) are fewer than 80 in both siCtr and siGCN2 samples, which are not consistent with the pooled cell numbers that the authors listed in the legend: n= 55, 30, 19, 19, 24 for siCtr; 55, 24, 33, 22, 26 for siGCN2.

6. As the authors noticed, "GCN2 is known as a translation regulator through phosphorylation of eIF2α" (line #112), so it was plausible to check whether translation of transcripts encoding mitotic regulators is altered in siGCN2 cells. However, the OPP-based global translation measurement they employed was too rough, it certainly would miss subtleties in changes of protein synthesis occurring uniquely in siGCN2 cells, given that potential GCN2-affected regulators likely account only dozens at the most. The best way to examine or identify the potential mitotic regulators affected by GCN2 is to perform ribosome profiling coupled with quantitative deep sequencing, or OPP-labeling coupled with mass spectrometry analysis as previously described (DOI:10.7554/eLife.07957; DOI:10.1073/pnas.1707514115).

7. Fig S3 C, D: I do not think that the blot shown in Fig. S3C is representative for quantified data shown in Fig. S3D. Which time points in Fig. S3C and S3D correspond to mitosis? Also, quantifications in Fig. S3D should have p values calculated and indicated.

8. Figure 4D: How much comparable between cell lysates used as input (lane #2) and immunoprecipitated sample (lane #3)? In other words, what is the percentage of cell lysate equivalent to that used for immunoprecipitation?

9. Figure 4E: It seems that siGCN2 also affects the total protein levels of PP1α and PP1γ in prometaphase-arrested cells?

10. In both Figure S5D and Figure 4D, E, HeLa cells were used to examine the phosphorylation status of PP1γ, why there is considerable PP1 phosphorylation in the input in Figure S5D but less so in Figure 4E?

11. Although the results based on both the in vitro phosphorylation assay and detection of in vivo phosphorylation of PP1α and PP1γ support the idea that these two phosphatases are likely phosphorylated by GCN2, it cannot exclude the possibility that GCN2 may only aid but not directly phosphorylate PP1α and PP1γ in cells. The in vivo phosphorylation sites on PP1α and PP1γ should be mapped, and the phosphorylation site mutants need to be characterized before the authors make any claim that GCN2 would actually phosphorylate PP1α and PP1γ in cells during prometaphase.

12. Figure 5: the presented data in Figure 5 are not described in the same order as those in the text. The representative images of mitotic cells stained for CENPE, phosphorylated Aurora B, and chTOG are in poor quality. Since the localization of these regulators upon siGCN2 is not simply "sustained" or "lost", counting the cells displaying the aberrant localizations is not the best way to characterize the far more complicated phenotypes caused by siGCN2. Also, the category of "aberrant localizations" is not clearly defined in the text or figure legend.

13. Considering that phosphatase PP1 in cultured human cells is also required for spindle checkpoint silencing as previously reported (DOI: 10.1083/jcb.201001006; DOI: 10.1038/ncb3065), I am curious whether compromised GCN2 function would also lead to spindle checkpoint silencing defects? This has not been, but should be, examined in this study.

Minor points:
1. In general, the text in this manuscript needs to be edited for English grammar and syntax.

2. Abstract, line #12-15: "In the absence of GCN2 function timing and levels of phosphorylation on key mitotic players is altered; leading to aberrant chromosome alignment, missegregating chromosomes, elevated number of tripolar spindles, and a delay in progression through mitosis.", should read as "In the absence of GCN2 (function), timing and levels of phosphorylation of key mitotic players are altered, which lead to aberrant chromosome alignment, missegregated chromosomes, elevated number of tripolar spindles, and a delay in mitotic progression.".

3. Line #22: To stay consistent with the title of the manuscript, better read "GCN2/eIF2αK4" as "eIF2αK4/GCN2".

4. Line #41: "Mitosis is driven by the mitotic spindle" does not fully reflect the current understanding on major principles of regulation of mitosis.

5. Line #80: "NOT" should be in lower case rather than capital letters.

6. "AURKA" and "AURKB", better to be named as "Aurora A" and "Aurora B" respectively, since they the commonly recognized names for these kinases.

7. In supplemental figures, it labels Fig. S1 as Figure EV1. Those labels should be kept consistent throughout the manuscript, this is applied to other supplemental figures as well.

8. Line #125: "To further test a rolw for GCN2-controlled translation, ..." should read "To further test a role for GCN2-controlled translation, ...".

9. Figure 8 legend and labels on graphs: Use of G0, G2, G3 or A0, A20, A30 to indicate the concentrations of GCN2 inhibitor or Aurora A inhibitor is very confusing. Please consider other straightforward labels instead.

Point by point response to the reviewers' comments

We would like to thank all the reviewers for their positive comments, constructive feedback and suggestions. We believe that the changes introduced in response to your assessment have considerably improved the manuscript.

Referee #1:

The manuscript by Stonyte and colleagues reports novel and potentially biologically impactful results, that ascribe a novel role for the amino acid sensing cytoplasmic kinase GCN2. The data supports a role for GCN2 in cell cycle progression through mitosis, which surprisingly is distinct from its major role as a sensor for amino acid availability and regulator of translational control of gene expression. The paper is well written, and the data is, for them most part clear and supports the main conclusions of the paper. The experimental design is robust with multiple biological and technical replicates and frequent use of rescue experiments using wild-type and mutant constructs to demonstrate biological specificity. However, there are some concerns with respect to some of the experimental results, especially with those pertaining to the biological consequence of GCN2 inhibition in determining cell fate.

Major issues:

1. An important question that arises from these results, is why there is a lack of any overt developmental phenotype in GCN2 knockout animals. GCN2-/- mice are viable, fertile and do not present any developmental abnormalities in utero or after birth (they do show some hepatocellular effects under specific diets). Given the important role in mitotic entry and progression in unstressed conditions shown here, one would expect significant defects in organ formation in rapidly growing tissues. The authors need to address this paradox.

Importantly, our data argue that GCN2 is not important during unstressed mitoses, but only during stressed mitoses characteristic of cancer cells. This is supported by three lines of evidence:

1. depletion or inactivation of GCN2 only leads to minor delays in mitotic progression in non-transformed cells - hTERT-RPE1 and BJ cells (Fig EV1, Fig 7, and new Fig panel EV5B). Despite a minor delay, these mitoses complete successfully and are not associated with any significant increase in mitotic segregation defects or micronuclei (Fig 8B).
2. In contrast, perturbation of GCN2 in various cancer cell lines – HeLa, U2OS, and MCF7 leads to mitotic delay (Figure 1, 7, EV1) and formation of micronuclei (Fig 8E)
3. GCN2 becomes essential for mitosis in non-transformed cells (RPE1 and BJ) that experience mitotic stress by inhibition of Aurora A (Fig 8B, Fig 9C, D, F, EV5B)

In response to the referee's comment and our new data, we have further clarified this message throughout our manuscript.

In a large-scale CRISPR screen it was found that about 13% of cancer-derived cell lines depend on GCN2 for survival when grown under unstressed conditions (cut off of dependency score $\leq$ -0.3) (Saavedra-García et al, 2021; Tsherniak et al, 2017). Even if we take a less

stringent cut off for GCN2 dependency (dependency score ≤ -0.1), only about 35% of cancer cell lines (1086 cell lines) are dependent on GCN2 (depmap.org), implying that most cell lines are not dependent on GCN2; consistent with our results obtained using the non-transformed hTert- RPE1 cells and fibroblast cells (new data Fig EV5B). Our data spurs on follow-up studies to reveal the underlying reasons why 13 % of cancer cells depend on GCN2 (e.g. genome instability, aneuploidy, synergizing mutations?), but we believe these are outside the scope of this work.

In summary, we show that GCN2 is essential only in cells that experience significant mitotic stress, under which conditions GCN2 regulation of PP1 activity becomes an essential means to delay mitotic progression. This would also explain why GCN2 -/- mice are viable as those animals do not experience the mitotic stress burden characteristic of many cancers.

2. The cells, though untransformed, are not "normal". The authors should test the effects of GCN2 inhibition (preferably with GCN2i) in human normal diploid fibroblast or epithelial cells.

We thank the referee for his/her comment. To address this, we have performed additional live-cell imaging experiments in normal fibroblasts (BJ cells). These new data confirm and extend our previous observations in RPE1 cells, showing that inhibition of GCN2 (GCN2i) is only essential for mitotic progression in “normal” cells when experiencing mitotic stress imposed by Aurora A inhibition. We have included these new data as Fig EV5B.

3. The immunoblot in Fig. 3A shows highly variable levels of total GCN2 which confound the results. Have the authors tried knocking GCN2 out completely using CRISPR and then transfecting with the different CGN2 constructs? That might achieve a more equal level of expression of total GCN2. Also, the legend indicating a "horizontal line" in the blot should be corrected to a "vertical line".

Our wild-type siRNA-resistant GCN2 allele exploits the same lentiviral backbone with the same transgene promoter as used for the mutants, and we have not found promoters that better mimic endogenous levels. As all GCN2 alleles are expressed at levels higher than endogenous GCN2 levels, this argues against too low transgene expression levels as a cause for lack-of-rescue of phenotypes following GCN2 depletion. This argues that comparison of phenotypes and impacts on mitotic progression is valid. Since the variability lies in transgene expression levels rather than in inefficient knockdown, we do not expect that repeating the experiments in a GCN2 knock-out background would lead to different conclusions.

The legend has been corrected.

4. The results in Fig 5 are not convincing, at least as explained in the text. It is stated that GCN2 inhibition changes the localization of AURA and other centrosome proteins form a localized to a diffuse phenotype. Yet, in Fig. 5A it is shown that in the second set of siGCN2 KO cells, AURA remains highly localized. Same goes for the results in Fig. 5B for ChTOG.

We have amended the text to make the localization patterns clearer.

We do not claim that the localization of Aurora A-P changed after knocking down GCN2, on the contrary. The antibody recognizes both Aurora A-P and Aurora B-P. While the centrosomal localization of Aurora A-P does not change upon GCN2 depletion, the Aurora B-P staining is more diffuse. We have also amended the text to clarify this in the relevant sections of the manuscript.

5. The immunoblot in Fig. 7D lacks any background. It needs to be reproduced with background shown, as well as quantitation of normalized band densities.

These antibodies are very good and we do have very little background without any image processing. We have provided source files with long exposures where some background can be seen. Quantifications are also provided (Fig 7E).

6. The results in Fig.8 are among the most biologically meaningful results of this work; yet, they are far from convincing. First, in terms of quantitation of the number of cells with micronuclei (panel E), there is no statistical comparison between the different groups, especially between the combined Aurora A and GCN2 inhibitors. Does the combination produce significantly higher number of cells with micronuclei than either inhibitor alone? More critically, there is no such comparison in panel I, which shows the impact on cell survival. This is an important point, since the authors emphasize the possibility of using this approach (synthetic lethality) in pathological conditions of GCN2 overactivation (e.g., tumors). Moreover, more robust apoptotic/survival assays need to be employed here to demonstrate proof-of-concept. Staining for Cleaved Caspase-3 and clonogenic survival following treatments with either inhibitor or combined treatments are warranted.
We have added additional figure panels (Fig 9E, F; Appendix Figure S5, 6) showing staining for activated caspase, which correlates well with the Hoechst staining. We have also performed clonogenic assays (Fig 9C). Statistical analyses are provided for Fig 8E,F as well as 9C and D, which show that the number of cells undergoing mitotic slippage, though not the number of cells with micronuclei, is significantly higher, and cell survival is lower as measured with either assay after combination treatment in both cell lines.

Minor points.
1. Fig. 1A needs a legend to explain what each different row of images represents (like in Fig. 1F below).
The legend has been modified.
2. The legend in Fig. 1D is rather confusing. Recommend changing "GCN2wtSiCtr" to "siCTR-GCN2wt". Same for GCN2wtsiGSN2.
We have changed the legends as suggested and also added “siR” as applicable.

Referee #2:

1. Does this manuscript report a single key finding? YES

2. Is the reported work of significance (YES), or does it describe a confirmatory finding or one that has already been documented using other methods or in other organisms etc (NO)? YES

3. Is it of general interest to the molecular biology community? YES

Suggests new substrates and pathway interactions in highly conserved cell processes which may impact on cancer therapeutic development.

4. Is the single major finding robustly documented using independent lines of experimental evidence (YES), or is it really just a preliminary report requiring significant further data to become convincing, and thus more suited to a longer¬format article (NO)?

YES - with reservations (see below)

Stonyte et al. show a novel finding, namely that GCN2 locates to the nucleus during mitosis, and that GCN2 activity is necessary for timely and correct mitosis. The strongest aspects of the manuscript are the initial observation of mitotic deregulation upon GCN2 silencing, GCN2 co-precipitation with PP1 and phosphorylation of PP1. However, there are significant shortcomings throughout the manuscript with a lack of care over typos, figure errors, controls etc. The manuscript could strongly benefit from a re-examination and correction from the authors. Having said that however, the experiments have been conducted rigorously, and the conclusions are compelling. The finding that inhibition of GCN2 may impact cell cycle progression, and this might be compounded in cancerous cells, is likely to be of interest to a broad readership.

Major Comments

A1 In general, the silencing of GCN2 through siRNA seems to be incomplete. the supplementary data figure (EV1) shows that there is a very poor silencing of GCN2, as there remains significant amount of GCN2 in their HeLa cells post-silencing (~70% of wt). Could the authors comment on the apparent disconnect between their western blot data (Figure 1C) and the data presented in EV1.

There is indeed some discrepancy between knock-down efficiency as measured by westerns and IF at the centrosome. As measured by western, we estimate GCN2 depletion to be higher than 90% while the reduction in the GCN2 signal by IF is closer to 50%. Both biological and technical reasons could be responsible for the discrepancy. As for biological reasons, we hypothesize that the protein has high affinity to the centrosome in mitotic cells and thus any remaining protein after knock-down accumulates at this location. Possible technical reasons include that high background can pose more of an issue in IF. Finally, it should be noted that two different antibodies were used in IF and immunoblotting experiments, which might also affect the results.

Interestingly GCN2 gets phosphorylated at the same time when it can be seen at the centrosome, suggesting that centrosomal GCN2 is phosphorylated. It should be emphasized that this is a speculation at this stage since we have not been able to find an antibody specific for the phosphorylated form to perform IF.

Given the referees' concerns regarding GCN2 localization by immunofluorescence (see also the response to Reviewer 3's comment 1 and comment 4), we have chosen reduce and nuance all statements and conclusions concerning the centrosomal localization in the Results and Discussion, and moved this figure panel to Appendix Fig S1.

We have extensively tried to generate GCN2 fusions with fluorescent tags at the C- or N-termini, but these constructs do not rescue GCN2kd as well as untagged GCN2 transgene, indicative of perturbed function of tagged GCN2. We will continue looking into the regulation of GCN2 activation and localization during mitosis in follow-up work.

A2 Supplementary Videos are insufficiently described and labelled. On Line 99, the authors refer to supplementary videos in an imprecise manner which limits their utility. If the videos are to be included in the manuscript, then the authors should annotate them sufficiently. For example, there are three videos labelled A0 G0, which is extremely ambiguous.

More detailed labeling is provided.

A3 The authors chose to activate GCN2 using UV irradiation of cells. This is problematic on several levels. Firstly, although GCN2 has been shown to be activated through UV exposure, it is perhaps the least 'canonical' route to GCN2 activation, and the mechanism is very much poorly understood. Secondly, UV irradiation is almost certainly going to cause a DNA damage response, and this may account for the perturbation of mitosis. Thirdly, although the authors make use of Halofuginone in EV3 (the standard compound for GCN2 activation), this is not the method of choice in Figure 3, which leads to a disconnect in the data.
We were using UV and HFG only to validate the mutants, but never in experiments focused on assessing and scoring mitotic phenotypes. We have used both stressors to confirm the results. In the revised manuscript we have replaced Fig 3A (UV treatment in the original manuscript) with data using HFG.

A4 There has been suggested to be significant overlap between GCN2 and PERK, and a functional redundancy in the signalling pathway when either is inhibited. Have the authors explored whether PERK can account for the delay, as opposed to the arrest, or mitosis?
To address this point, we have performed live-cell imaging experiments to monitor progression through mitosis in the presence of a PERK inhibitor (Fig EV5A) and assessed PP1 phosphorylation in mitosis in the absence of PERK activity (Fig EV5B). The results indicate that PERK cannot perform the mitotic function of GCN2.

A5 Figure EV3 B and C, Figure EV4A, EV6A, 6A, 6F, - Loading Controls are missing for blots. In general, the quality of the blots is sufficient, but these controls are necessary. Would the authors be able to provide loading controls for all blots?
We have provided total-protein loading controls for each phosphorylated protein (GCN2 for GCN2-P, eIF2α for eIF2α-P in figures EV2, EV3, EV5; CENPE and Aurora B for the phosphorylated proteins in fig EV4, TACC3 and Aurora A in Fig 6A, total proteins for every phosphorylated protein in fig 6F). We have now added γ-tubulin or CDK loading controls as well.

A6 Have the authors assessed whether GCN2AAXA is active or correctly folded? Does treatment with Halofuginone still elicit an ATF4 phosphorylation/autophosphorylation response?
In our revised manuscript we have added new data to show that ATF4 is induced upon HFG treatment in the GCN2^{AAXA} mutant (Fig EV3B). This supports the notion that GCN2^{AAXA} retains its activity.

A7 The authors use an extremely narrow range of GCN2i - between 1 and 3 micromolar. Do they have data supporting this range of concentrations, showing that at 3 micromolar that GCN2 is entirely inhibited and no longer phosphorylates eIF2alpha on exposure to halofuginone?
When assessing mitotic phenotypes, our aim was to use GCN2i concentrations that are not unnecessarily high, in order to reduce the chance of off-target effects. We tested autophosphorylation both after HFG treatment and after UV irradiation in the presence of the inhibitors; 2 µM efficiently, though not completely inhibits GCN2, while 3 µM is somewhat more efficient. We have also included new data showing ATF4 induction at different GCN2i concentrations in starvation experiments (Fig EV5A), illustrating the effective inhibitory effect on GCN2 activity.

A8 In most anaphase timing experiments; the x axis extends to 150 minutes. However, in Figures 2C, 7A and 7B this is not the case.
The scale of the x axes has been changed.
This extends to a wider point - how was significant deviation for anaphase timing calculated? Is the significance calculated at the 150-minute timepoint, or the midpoint of the curve?
We used the Kolmogorov Smirnov test, which compares plot curves along the trajectory and is generally used to assess cumulative frequency distributions. This information is also added in the manuscript.

Minor Points:

As previously mentioned, the manuscript has many typographical errors. Here is a selection.

B1 Line23: The statement requires adequate citation.
B2 Line28: The statement requires citation.
Citations are inserted.

B3 Line72: "few phosphatases stand for the removal of these phosphates". I am not certain as to the meaning of this phrase - consider rewording
We have changed the sentence to “few phosphatases are responsible for the removal of these phosphates”.

B4 Line80: Capitalisation of "NOT" is unnecessary.
Corrected

B5 Figure 1D. The outline of siGCN2 significance bands is a different colour to the main line, which is confusing.
The colours have been changed on all graphs showing live-cell imaging data.

B6 Line 125 Typo "rolw"
Thank you! Corrected.

B7 None of the cell sorting data figures have sufficient details for axes.
The figures have been changed as suggested.

B8 Figure EV3 C - Please specify the antibody used for GCN2-P (i.e. which site is being probed)
The antibody is now specified in the Methods section.

B9 Line 125-127 - "downstream consequences" rephrase as ambiguous
We have changed the text to “used ISRIB, a drug that binds eIF2B and thereby antagonizes the inhibitory effect of phosphorylated eIF2α”

B10 Figure 3A - "The vertical line on the GCN2 blot indicates that the bands shown for the non-transduced cell line ("wt") are a longer exposure of the same blot." If I follow correctly, these are two expsoures, both in wt cells. But the loading controls remain the same? How is that possible?
We apologize for the unclear labeling. The “wt” labeling has been removed to avoid confusion whether it refers to the endogenous or the transduced siR allele.

All our lentiviral constructs are expressed to higher levels than the endogenous protein, which made it necessary to show two different exposures for GCN2. The labeling has been changed so that cells expressing the siRNA-resistant protein are labeled siR.

B11 Line 152 Typo "GNC2"
Thank you! Corrected.

B12 Clarity is required for when the authors are silencing the endogenous GCN2 (siGCN2) or using the silencing resistant GCN2. The use of GCN2wt to denote the use of a silencing resistant GCN2 expression is very confusing.
In an attempt to make the nomenclature clearer, in the graphs and immunoblots we have changed the labeling to siR to denote the siRNA-resistant cell lines, and added the mutations (eg K619R) as appropriate.

B13 Figure 4A typo - "Bovin"
Thank you! Corrected.

B14 The term in vitro is inconsistently italicised throughout.
We have italicised the term throughout.

B15 Figure 7C - labels on x axis are poorly formatted.
The labeling has been changed.

B16 Figure 7 Figure legend. Typo "GNC2"
Thank you! Corrected.

B17 Figure EV6C - x axis labels oddly spaced
This is not easy to change. The time course involved a long period where we did not take samples. In the interest of space, the plot is a column plot rather than an xy plot, and thus the x axis labels are not to scale.

B18 Figure EV7B/D Either 7,5 or 7.5 - not both.
Thanks! They are changed to 7.5.

B19 References are highly variable in format.
We have re-formatted the references using EndNote.

Referee #3:

In this manuscript, Stonyte et al. investigated a potential function of GCN2 that is independent of its canonical role in eIF2α phosphorylation and translation regulation, although GCN2 has been generally regarded as a protein translation regulator based on its kinase activity toward eIF2α. In this manuscript, the authors show that GCN2 can localize at centrosomes during mitosis, and likely this pool of GCN2 is involved in the regulation of mitosis by phosphorylating two substrates PP1α and PP1γ. Indeed, both PP1α and PP1γ can be phosphorylated by GCN2 in in vitro kinase assay, and their phosphorylation status is also altered in vivo when GCN2 is depleted. They also show that pharmacological GCN2

inhibition leads to mitotic errors, which renders synergistic effect with Aurora A inhibition. These findings shed light on the unappreciated roles of GCN2 stress kinase in mitotic fidelity. In principle, dissecting if/how eIF2aK4-GCN2 stress kinase regulates PP1 would be of interest, however, this study only establishes the connection but does not conclusively demonstrate this. The authors should address the following points prior to publication.

Major points:

1. The title of "Figure 1. GCN2 depletion leads to a delay through mitosis." does not represent the whole conclusion of the data shown in this figure, because the data also showed that GCN2 is localized at centrosomes during mitosis, which is a novel observation.

Given the concerns regarding the centrosomal signal (see the response to Reviewer 2's comment A1, and comment 4 below), we moved this figure panel to Appendix Fig S1.

2. Figure 1B and 1D: How the time point 0 min was defined? The authors did not explain how this was done in either the main text and the legend for the figure, or in the Methods section. What criteria were used, for example, the markers for prophase, metaphase, or anaphase? I guess these experiments were performed with n = 6 or 5 cells for each sample, namely the sample size, rather than n = 6 or 5 independent experiments respectively. If this is true, then I would think that the sample size was pretty small. In addition, I strongly suggest the authors reselect the color for each chart/sample, because the current colors used are difficult to be distinguished by readers. The same issue is applied to Figure 3B and 4C.

For each cell t=0 is prophase as judged by the first frame showing chromatin condensation. Anaphase was scored based on chromosome separation. This information is now provided in the Methods section as well as in the figure legends.

The colours have been changed.

All live-cell imaging experiments were performed with 3-6 independent time courses. The number of cells counted in each time course was typically 50-100, but in some samples (especially when there was a long mitotic delay, or in untransformed cells where fewer cells go through mitosis) it was around 30. The number of cells counted in each replicate is shown in the Appendix. In the manuscript we added the total number of cells analyzed in all the replicas pulled together in each figure legend.

3. Figure EV1 (should be Figure S1): It seems the depletion of GCN2 at centrosomes is rather incomplete based on the representative images authors provided, but in Figure 1C, the GCN2 depletion seems rather complete.

4. I wonder how specific the anti-GCN2 antibodies are? To definitely show or confirm the localization of GCN2 at centrosomes, I would suggest the authors to employ CRSPR-Cas9 to knock-in GCN2-GFP, thus avoid the specificity issue of immunofluorescence.

5. In Figure S1B, the scattered dots shown presumably represent the relative fluorescence intensities of individual GCN2 signals in each cell measured, but the numbers of measured GCN2 signals (based on depicted dots in the graph) are fewer than 80 in both siCtr and siGCN2 samples, which are not consistent with the pooled cell numbers that the authors listed in the legend: n= 55, 30, 19, 19, 24 for siCtr; 55, 24, 33, 22, 26 for siGCN2.

As indicated above (referee 2, comment A1) we have tried to tag GCN2 at the N- and C-terminus, but fusion with any fluorescent tag we have tried renders the GCN2 kinase inactive. We have also tagged the protein on the N terminus with three different tags, but none of these constructs rescues the mitotic phenotype. GCN2-Flag is functional, but we cannot detect it in IF at centrosomes, which we attribute to reduced epitope availability.

Since we have not been able to strengthen and corroborate our data regarding the centrosomal localization of GCN2, we have chosen to reduce and nuance all statements and conclusions emphasizing this in the Results and Discussion. The figure showing the cell-cycle specific localization is moved to Appendix Fig S1.

Figure EV1B, now Appendix Fig S1C: yes, the dots represent intensity values in individual cells and this information is now added to the figure legend. The figure is changed to show data from each experiment separately.

6. As the authors noticed, "GCN2 is known as a translation regulator through phosphorylation of eIF2α" (line #112), so it was plausible to check whether translation of transcripts encoding mitotic regulators is altered in siGCN2 cells. However, the OPP-based global translation measurement they employed was too rough, it certainly would miss subtleties in changes of protein synthesis occurring uniquely in siGCN2 cells, given that potential GCN2-affected regulators likely account only dozens at the most. The best way to examine or identify the potential mitotic regulators affected by GCN2 is to perform ribosome profiling coupled with quantitative deep sequencing, or OPP-labeling coupled with mass spectrometry analysis as previously described (DOI:10.7554/eLife.07957; DOI:10.1073/pnas.1707514115).

We completely agree that a possible involvement of GCN2 in cell-cycle specific translation is a very interesting possibility. Certainly, the OPP-based global translation measurements do not allow us to exclude that translation of selected transcripts is regulated in a GCN2-dependent manner and this possibility was pointed out in the manuscript. However, we argue that the experiments with ISRIB support the idea that the translation regulation GCN2 might be responsible for is not the reason for the mitotic phenotypes. We believe that a comprehensive characterization of GCN2-dependent translation regulation in mitosis (or lack of therein) is fraught with experimental challenges and is outside the scope of this manuscript. The main difficulty we foresee with both suggested approaches is that cell-cycle synchronization is required. However, any synchronization method causes stress (Anda & Grallert, 2019; Stonyte et al, 2018), which will complicate interpretation of data when inhibiting a stress-response kinase. As for the OPP-mass spec approach, a labeling time of 2 hours was used in the paper referred to by the reviewer, which is not compatible with cell-cycle analyses during mitosis.

However, to start addressing this intriguing question, we have performed experiments to assess the translation rates of selected mitotic proteins +/- GCN2i. We have chosen mitotic proteins whose selective translation under some condition, even if not in the context of cell cycle, has been shown (Fujita et al, 2021; Lai et al, 2017; Stumpf et al, 2013; Tanenbaum et al, 2015). Intriguingly, while mitotic translation of most of the selected proteins is not affected by GCN2, PP1γ accumulates to higher levels in the presence of the GCN2 inhibitor (Fig 2D, E). Remarkably, this increase cannot be observed in cells treated with ISRIB (Fig EV3E, F), suggesting that it does not occur through the canonical pathway involving eIF2α phosphorylation. Instead, it might reflect a difference in PP1γ protein stability in cells treated with the GCN2 inhibitor. In conclusion, GCN2-dependent, but not eIF2α-P-dependent, regulation of PP1γ levels might contribute to the mitotic phenotype and this is now stated in the manuscript.

7. Fig S3 C, D: I do not think that the blot shown in Fig. S3C is representative for quantified data shown in Fig. S3D. Which time points in Fig. S3C and S3D correspond to mitosis? Also, quantifications in Fig. S3D should have p values calculated and indicated.

To show the timing of mitosis, the blot was reprobed with an antibody specific for phospho-Aurora A and B, which serve as mitotic markers. The quantifications are from six independent experiments. No single blot shows the average of the six. p values (calculated with Anova) are stated in the figure legend.

8. Figure 4D: How much comparable between cell lysates used as input (lane #2) and immunoprecipitated sample (lane #3)? In other words, what is the percentage of cell lysate equivalent to that used for immunoprecipitation?
The phosphorylation is reflected in the ratio of the upper and lower bands and we are not comparing band intensities from lane to lane. Therefore, in these experiments we did not focus on the efficiency of the IP-s as long as we pulled down significant amounts of protein. The "input" is 0.5% of the total lysate. The rest of the IP is used for the kinase assays. We did not quantify how much material was lost during the washing steps. About 30 % of the samples is loaded on the phos-tag gels.

9. Figure 4E: It seems that siGCN2 also affects the total protein levels of PP1α and PP1γ in prometaphase-arrested cells?
Yes indeed, we will continue looking into these observations in follow-up work. However, it is difficult to conclude from the phos-tag gels because these blots cannot be reprobed to detect a loading control. The new data addressing the effect of GCN2 on translation in mitosis suggest that PP1α translation is not affected by inhibition of GCN2, whereas PP1γ translation is affected (Fig 2D, E, Fig EV2E, F).

10. In both Figure S5D and Figure 4D, E, HeLa cells were used to examine the phosphorylation status of PP1γ, why there is considerable PP1 phosphorylation in the input in Figure S5D but less so in Figure 4E?
This can be attributed to some normal experimental variations. Experiments were done on different days, meaning a different batch of cells, buffers and inhibitors; as well as small differences in time during which the lysates were left on ice after harvesting. However, in each experiment the same IP was split and used in the kinase assays +/- GCN2 (Fig 4D and EV5D). In Fig 4E lysates were prepared and boiled in sample buffer immediately. The impact of this treatment versus the fate of the IP-s is most obvious on Fig 4D where the lysates prepared by boiling immediately "prometaphase" and lysates prepared for IP-s "input" are shown side by side. In Fig EV5D (EV4E in the revised manuscript) we also used λ phosphatase before performing the kinase assays, so that any phosphorylation carried over from the input was removed.

11. Although the results based on both the in vitro phosphorylation assay and detection of in vivo phosphorylation of PP1α and PP1γ support the idea that these two phosphatases are likely phosphorylated by GCN2, it cannot exclude the possibility that GCN2 may only aid but not directly phosphorylate PP1α and PP1γ in cells. The in vivo phosphorylation sites on PP1α and PP1γ should be mapped, and the phosphorylation site mutants need to be characterized before the authors make any claim that GCN2 would actually phosphorylate PP1α and PP1γ in cells during prometaphase.
This is indeed a very interesting question that is challenging to answer conclusively. Our data show a GCN2-dependent phosphorylation in vivo and the in vitro kinase assays make a strong case for this indeed being direct. Having said this, we cannot completely exclude a scenario where GCN2 can phosphorylate PP1α and PP1γ in vitro but regulates this indirectly in vivo.

We have added new data (Fig EV3D) showing in vitro kinase assays on PP1α using GCN2 immunoprecipitated from starved cells (as we did in the original manuscript using PP1γ as a substrate, Fig EV3C in the revised manuscript).

We have tried to perform mass-spec analyses to identify the phosphorylation sites, but have been unsuccessful so far in indentifying the relevant peptides, let alone their phosphorylation status (two different mass spec facilities and three different enzyme-digestions). We have turned to genetics and made a number of TA and SA substitutions at sites that are known to be phosphorylated by other kinases or have been predicted to be phosphorylated. The investigation of these mutants requires more detailed analyses and dissection, and could therefore not be completed within the timeframe of these revisions.

We have found that the PP1γS311A mutant loses the bandshift. This site is known to be phosphorylated by CDK1. GCN2 alone cannot maintain the bandshift of PP1γ in vitro when CDK1 is inhibited, suggesting that they do not simply compete for the same site and phosphorylate different pools of PP1γ. The phosphorylation status of CDK substrates and several PP1γ substrates is affected differently by CDK inhibition and by GCN2 inhibition. These results are compatible with several different models; (i) GCN2 might be able to phosphorylate PP1γ only when it is already phosphorylated by CDK1; (ii) PP1γ exists in different subcomplexes and the two kinases can bind different subsets of these complexes. (iii) GCN2 affects PP1 phosphorylation through CDK1. We cannot exclude the latter model, but again, the in vitro kinase assay using recombinant GCN2 argues that GCN2 can phosphorylate PP1γ making this the most parsimonious interpretation.

In light of these alternative models, we rephrased the suggestion “GCN2 phosphorylates PP1” to “PP1 is phosphorylated in a GCN2-dependent manner”. We believe that adding our current, somewhat preliminary data would not enhance the manuscript, and a complete deciphering of the mechanism is beyond the scope of this work.

12. Figure 5: the presented data in Figure 5 are not described in the same order as those in the text. The representative images of mitotic cells stained for CENPE, phosphorylated Aurora B, and chTOG are in poor quality. Since the localization of these regulators upon siGCN2 is not simply "sustained" or "lost", counting the cells displaying the aberrant localizations is not the best way to characterize the far more complicated phenotypes caused by siGCN2. Also, the category of "aberrant localizations" is not clearly defined in the text or figure legend.
We have amended the text to better explain what “aberrant localizations” refer to. The order of the images has been corrected.

13. Considering that phosphatase PP1 in cultured human cells is also required for spindle checkpoint silencing as previously reported (DOI: 10.1083/jcb.201001006; DOI: 10.1038/ncb3065), I am curious whether compromised GCN2 function would also lead to spindle checkpoint silencing defects? This has not been, but should be, examined in this study.
This is indeed a very interesting question. In new experiments we have shown that the mitotic delay observed in the absence of GCN2 function depends on the SAC, because inhibiting the SAC kinase Mps1 or knocking down MAD1L1 abolish the delay (Fig EV4F). This finding suggests that GCN2-dependent inhibition of PP1 is not essential for the SAC, but does not exclude that GCN2 has some role in the SAC. In Fig EV4G we have addressed this further. We reasoned that failure of an early function of GCN2 might mask an involvement in the SAC, but if mitosis can progress past this early function with GCN2 activity in place, a role in the SAC would be revealed upon subsequent GCN2 inhibition. To address this scenario, HeLa cells were synchronized by thymidine block-and-release, and then progression through

mitosis was followed by live-cell imaging. GCN2 inhibitor or an Mps1 inhibitor was added halfway through the mitotic peak. Cells that entered mitosis up to 15 min before receiving the Mps1 inhibitor spent a shorter time in mitosis than cells that entered mitosis earlier in the time course, consistent with its known role in the SAC. In contrast, cells that entered mitosis up to 15 min before GCN2 inhibition progressed through mitosis with normal timing, while cells that entered mitosis just before or at the time of adding GCN2i delayed entry into anaphase (Fig EV4G). These data suggest that GCN2i does not interfere with the SAC.

Minor points:
1. In general, the text in this manuscript needs to be edited for English grammar and syntax.
2. Abstract, line #12-15: "In the absence of GCN2 function timing and levels of phosphorylation on key mitotic players is altered; leading to aberrant chromosome alignment, missegregating chromosomes, elevated number of tripolar spindles, and a delay in progression through mitosis.", should read as "In the absence of GCN2 (function), timing and levels of phosphorylation of key mitotic players are altered, which lead to aberrant chromosome alignment, missegregated chromosomes, elevated number of tripolar spindles, and a delay in mitotic progression.".
Changed as suggested.

3. Line #22: To stay consistent with the title of the manuscript, better read "GCN2/eIF2αK4" as "eIF2αK4/GCN2".
Thanks! Changed as suggested.

4. Line #41: "Mitosis is driven by the mitotic spindle" does not fully reflect the current understanding on major principles of regulation of mitosis.
Yes of course; this paragraph is meant as a very short introduction to mitosis and the SAC, rather than an overview of the major principles.

5. Line #80: "NOT" should be in lower case rather than capital letters.
Corrected.

6. "AURKA" and "AURKB", better to be named as "Aurora A" and "Aurora B" respectively, since they the commonly recognized names for these kinases.
It has been changed as suggested.

7. In supplemental figures, it labels Fig. S1 as Figure EV1. Those labels should be kept consistent throughout the manuscript, this is applied to other supplemental figures as well.
The revised manuscript contains five EV figures in line with the instructions from the Journal, and the rest of the supplementary material is provided as Appendix files.

8. Line #125: "To further test a rolw for GCN2-controlled translation, ..." should read "To further test a role for GCN2-controlled translation, ...".
Thank you! Corrected.

9. Figure 8 legend and labels on graphs: Use of G0, G2, G3 or A0, A20, A30 to indicate the concentrations of GCN2 inhibitor or Aurora A inhibitor is very confusing. Please consider other straightforward labels instead.
The labels have been changed to Ctr, A, G and AG. Concentrations are provided in the figure legends.

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Dear Beata,

Thank you for submitting your revised manuscript for consideration by EMBO reports. I apologize for the delayed response, but it has now been seen by two of the original referees who agreed to re-evaluate it, and we have received their comments, which are included below.

As you will see, both referees find the revised version of the manuscript significantly improved, they mention that the added data and the textual improvement have strengthened the manuscript, and they now both recommend publication. There is only one minor suggestion of referee #3 regarding labeling in some Figure panels, which we need you to address in a revised manuscript (please see the details in the referee's report below).

From the editorial side, there are also a few things that we need from you before we can proceed with acceptance of the manuscript:

- Please provide up to 5 keywords in your revised manuscript.

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Best regards,

Ioannis

Ioannis Papaioannou, PhD
Editor
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Referee #2:

As I previously reported, the manuscript was technically strong and had clear observations which may be of interest to a wide readership. The additional data provided, and the increase in quality of the manuscript are of a sufficient level to recommend publication.

Referee #3:

The authors have carefully and comprehensively addressed and discussed all comments and concerns that were previously raised. The added data (controls and new experimental data) and text revision have improved the manuscript and increased the significance of the message, and the manuscript is ready for publication.

Minor point:
Figure 8, 9 and Figure EV5B: There are enough space in these figures to have more directly-speaking labels. I strongly suggest to have labels with ctrl, GCN2 inhibitor, and Aurora A inhibitor, or at least ctrl, GCN2i, and Aurora Ai, instead of ctr, G and A, which are pretty confusing.

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Department of Radiation Biology
The Norwegian Radium Hospital
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E-mail: beata.grallert@rr-research.no

Oslo, 16th May 2023

Dear Ioannis,

Thank you for the favourable decision on our manuscript!
In the revised version we have corrected the errors and provided missing information. We have also changed the labeling on the figure as suggested by Reviewer #3.
I hope nothing is overlooked and the paper is now ready for publication.

Best regards,
Beata

2nd Revision - Editorial Decision **22nd May 2023**

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Thank you for the submission of your revised manuscript and its associated files to EMBO reports. We have noticed that there are still a few remaining issues that I would kindly ask you to address in a new revised version:

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Best regards,

Ioannis

Ioannis Papaioannou, PhD
Editor
EMBO reports

The authors have addressed all minor editorial requests

Dr. Beata Grallert
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Montebello
Oslo, orcid||||||| 0310
Norway

Dear Beata,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Best regards,

Ioannis

Ioannis Papaioannou, PhD
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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 replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
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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.
- 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.
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