



Basal CHK1 activity safeguards its stability to maintain intrinsic S-phase checkpoint functions

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March 11, 2019

Re: JCB manuscript #201902085

Dr. Matthias Altmeyer
University of Zurich
Department of Molecular Mechanisms of Disease
Zurich 8057
Switzerland

Dear Dr. Altmeyer,

Thank you for submitting your manuscript entitled "Basal CHK1 activity safeguards its stability to maintain intrinsic S-phase checkpoint functions". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

Thank you for submitting your manuscript entitled "Basal CHK1 activity safeguards its stability to maintain intrinsic S-phase checkpoint functions". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that the reviewers overall found the report of basal CHK1 activity throughout the cell cycle preventing its destabilization and the dependence of this activity on ETAA1 intriguing. They did differ in their assessments of what the scope of a study that would be appropriate for JCB would be, with Reviewer #2 somewhat less enthusiastic. We discussed the overall feedback from the reviewers in depth. We do find your proposed model interesting and also agree with Reviewer #2 that a shorter format would indeed be more suitable for the publication of the results, in part because of the novelty of the concept of basal, continuous CHK1 activity across the cell cycle and its impact should it fall below a certain threshold in terms of replication stress and DNA damage. JCB Reports must provide definitive, highly novel findings of high interest to a wide audience of diverse cell biologists, with the potential to open up new avenues of research. We feel that the work could be appropriate for resubmission in the Report format, should you be in a position to thoroughly address the following points in order to strengthen the core claims of the report and ensure the definitiveness of the conclusions.

We feel that an important focus of the revision experimental efforts should be dedicated to tackling all the reviewers' points about the Figure 5 data connecting CHK1 basal activity throughout the cell cycle to the S-phase checkpoint and replication stress/damage in S phase. Revs#1 and #3 have direct experimental suggestions to strengthen the proposal that the checkpoint defect leading to low CHK1 activity would lead to CHK1 degradation, and so lower CHK1 activity, triggering at some point replication stress. We particularly feel that Reviewer #3 put it well and suggested an appropriate and interesting experiment by combining the low level of CHK1 inhibition with low dose DNA damage and looking at the level of DNA damage response, which would strengthen the physiological function of the basal CHK1 activity.

Additionally, all the reviewers felt that the TOPBP1/ETAA1 results need to be strengthened (Rev#1

second major point, Rev#2 point #3, Rev#3 point #3). We agree that these important studies providing notable mechanistic definition should be included and bolstered by experimentally tackling the reviewers' points rigorously. Rev#2 asked if the degradation of CHK1 observed upon CHK1 inhibition is reverted by CDK1 inhibition - a relevant point we agree should be straightforward to examine. Rev#3 recommended adding mutant analyses (#2, including to better understand the contribution of the kinase activity) -- these are interesting suggestions and phospho mutants could be quite useful. We encourage you to try these studies experimentally - ideally with knockins and not overexpression of the tagged mutants - as technically feasible.

Lastly, an important aspect of the revision will be to seriously consider and tackle the reviewers' points regarding data presentation. We would add that, in our own assessment of the work, we were concerned by the lack of statistical analyses to test for significance, in particular for the analyses of CHK1 levels. Adequate tests need to be added throughout the paper.

On the other hand, the reviewers suggested other experiments and had other concerns that may be addressable in the text. For instance, Rev#2 wanted more evidence supporting the conclusion that DDB1-mediated proteasomal degradation controls CHK1 levels during Chk1 inhibition. While we agree that the data in 3D show that the basal level of CHK1 is affected by DDB1 loss, we do not think that it precludes that DDB1 is involved in keeping it low after inhibition. It is possible that DDB1 is involved in maintaining a steady-state control of CHK1 levels -- teasing apart the detailed mechanisms underlying these observations seems beyond the scope of this Report, but this point should certainly be addressed in the manuscript text.

Rev#3 asked if CHK1 inhibition affects DNA replication since it is known that CHK1 loss alters DNA replication (point #1, see also #1 from Rev#2). Based on the data in Figure 2 showing that the EdU staining is not affected, doing DNA combing would be interesting but we do not feel that it would be essential for this paper. This would also address one of the minor points from Reviewer #3 about increased EDU intensity. There is a definite bump in the EdU but chasing that might not be helpful for this work. In minor points, Rev#3 asked about other hits from the initial screen -- we agree that this is an interesting question but likely beyond the focus of this particular Report.

Please let us know if you anticipate any issues addressing these points or would like to discuss the revisions further, we would be happy to discuss and clarify any point as needed.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for a Report is < 20,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Reports may have up to 5 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, <http://jcb.rupress.org/site/misc/fora.xhtml>. All figures in accepted manuscripts will be screened prior to publication.

*****IMPORTANT:** It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication.

Please ensure that you have access to all original microscopy and blot data images before submitting your revision.***

Supplemental information: There are strict limits on the allowable amount of supplemental data. Reports may have up to 3 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

Our typical timeframe for revisions is three months; if submitted within this timeframe, novelty will not be reassessed at the final decision. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to the Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Agata Smogorzewska, MD, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The authors have investigated Chk1 levels and importance by titrating Chk1 inhibitors to cells. They convincingly show that there is destabilization of Chk1 after Chk1 inhibition or depletion of upstream Chk1 regulators as ATR and ETAA1. Somewhat surprisingly, they find that this occurs throughout the cell cycle, and not only when Chk1 activity could be expected to be triggered during S-phase. They further propose that Chk1 activity regulating Chk1 levels could function as a safeguard to maintain genome stability: if the checkpoint regulation is faulty so that a basal Chk1 activity is not maintained, it could eventually lead to loss of Chk1 and massive DNA damage in S phase.

That there is destabilization of Chk1 after Chk1 inhibition is clear. Also that there is destabilization after depletion of upstream regulators as ATR and ETAA1. Chk1 destabilization after checkpoint inhibition has been noted earlier, but the clear merit here is that it is shown to be de-coupled from DNA damage. It is also interesting that Chk1 is shown to have a basal activity throughout the cell cycle. There is not a lot of mechanistic insight into how stabilization occurs though, but considering the central role of Chk1 for replication stress I think the auto-stabilization idea could be interesting to a wide audience interested in genome integrity.

Major points

A main point is that there is a vicious cycle (or negative circuit in abstract), in which a checkpoint defect leading to low Chk1 activity would destabilize Chk1, leading to even lower Chk1 activity, ultimately leading to RS. The main experiment in support of this is that siRNA-mediated depletion of Chk1 only eventually leads to p21/gH2AX in S-phase. Although the concept is plausible and interesting - and certainly should be included - I don't agree the experiments show the existence of such a cycle beyond doubt and think the text should be adapted to indicate it is an idea put forward by the authors. To show a vicious cycle it would be necessary to somehow decouple it - for example by adding low levels of Chk1 inhibitor to cells expressing a mutant Chk1 that is not destabilized by reduced activity. Getting such an experiment right could be a major investment and not absolutely necessary to put forward the idea. I would recommend to rather clearly indicate in the text that it is a concept proposed by the authors (mainly abstract, results, legend fig 5 - in discussion it's more balanced).

It is argued that TopBP1 does not contribute to maintain Chk1 levels, but the data for that in S4H is not convincing.

Chk1 levels from imaging are shown as violin plots rather than dot plots - which is fine, but should be complemented by raw data as it makes it difficult to assess cell cycle effects except in fig S2F. In that one it may seem as Chk1 is not de-stabilized in S-phase? (at 8h the S-phase cells have been through treatment in G1). A dot plot of Chk1 levels vs DNA should be included. Similarly, the RPE data is crucial to show that this is not restricted to cancer cells. The RPE data on Chk1 levels should be re-plotted to also show the levels throughout the cell cycle.

Reviewer #2 (Comments to the Authors (Required)):

In the present manuscript, Michelena et al present their work on the identification of a positive feedback loop by which CHK1 activity sustains CHK1 protein levels. The work is based on defining conditions by which a mild CHK1 inhibition leads to lower CHK1 levels, without significant DNA damage (Figures 1 and 2). The authors later show that CHK1 degradation in this situation is in part mediated by DDB1-guided proteasomal activity (which was known in the context of DNA damage) and ETAA1.

My overall impression of this work is that while showing that CHK1 activity sustains CHK1 levels is of interest, at this point the manuscript is a bit premature for publication. Some of the figures are redundant (Fig. 1/2), and others insufficient to support the claim made (Fig. 5). Altogether, I do not believe that this MS fits the standards that one expects from JCB.

Other comments/suggestions:

#1 Is the degradation of CHK1 observed upon CHK1 inhibition reverted by CDK1 inhibition? In other words, is this a consequence of a premature activation of the mitotic program, where many repair and replication factors are degraded?

#2 Since both MG132 and DDB1 depletion increase CHK1 levels even in the absence of CHK1 inhibition, it is difficult to prove that DDB1-guided proteasomal degradation is the mechanism that operates during CHK1 inhibition.

#3 The fact that TOPBP1 depletion does not affect CHK1 levels is difficult to understand. In contrast to ETAA1, TOPBP1 is essential at the cell level, and absolutely critical for ATR activity in all conditions. If the authors still want to make this case, they should perform solid genetic experiments to substantiate the dispensability of TOPBP1 in this phenomenon.

#4 Figure 5 is a rather indirect way to make their point. It simply provides several evidences of genomic instability upon ETAA1 depletion or sustained CHK1 inactivation, which I would guess is rather expected.

#5 In general, I think the work provided here is not substantial for an Article. Most figures are rather scarce in data, or redundant with previous figures. I would suggest the authors to consider shorter and more condensed formats for the publication of this work.

Reviewer #3 (Comments to the Authors (Required)):

The manuscript by Michelena J et al entitled "Basal CHK1 activity safeguards its stability to maintain intrinsic S-phase checkpoint functions" reports that the activation of Chk1 is required to maintain CHK1 steady-state levels in unchallenged cells. They also report that a reduction in CHK1 steady-state levels causes loss of the S-phase checkpoint, and compromised capability of cells to respond to RS.

While the manuscript is potentially interesting, the biological implications of the protection of Chk1 stability by Chk1 activation in undamaged cells was not directly addressed. Another weakness of the manuscript is the limited tools used. Simple tools such as a kinase-dead mutant, antibodies against ATR-dependent phosphorylation of Chk1 and DNA combing technology will enrich the model proposed by the authors.

Major points

1) It is very well established that Chk1 loss causes alterations in DNA replication parameters (namely origin firing and DNA elongation rates) which in turn trigger gamma H2AX accumulation and double-strand break formation. This work demonstrated that moderate CHK1 inhibition affects the levels of Chk1 but not gamma H2AX and markers of double-strand break formation. It would be informative to establish whether replication parameters such as origin firing and DNA elongation are affected in such experimental settings.

2) A Chk1 KD mutant should be used in this study. Basically, all experiments in Figure 3 should be performed with a Chk1 KD mutant. Accordingly to the model presented in this manuscript, the half-life of Chk1 KD should be shorter than the one of Chk1 wt because of increased proteolysis. Also, the use of Chk1 S345A and S317A mutants in half-life experiments and MG132-treatments may be informative.

3) The levels of pCHK1S345 and pCHK1S317 should be monitored, especially when assessing the effect of ETAA1 and TOPBP1 depletion in the context of unchallenged proliferation or CPT treatment. Also, as Chk1 levels are regulated in all phases of the cell cycle, the evaluation of 296, 345 and 317 in G1, S, G2 would be very informative.

4) The assessment of the biological relevance of the findings in this manuscript is peculiar. In my opinion, Figure 5 builds upon a different aspect of Chk1 biology which is exploring the consequence of Chk1 loss (which is quite well documented). The settings used in Figure 5 (siChk1) are, so far, dissociated from the main subject of Figures 1-4. It is unclear if the authors assume that the levels of Chk1 observed after 24 hours of siChk1 (shown in Figure 5C) recapitulate the ones observed

after 4 hours of low levels of Chk1 inhibitor (Figure 1). In any case, the link is confusing and so is the conclusion. Figure 1 indicates that, by controlling Chk1 activation, the levels of Chk1 can be modulated without affecting replication stress markers. In contrast, Figure 5 concludes that the relevance of maintenance of steady-state levels of Chk1 by Chk1 activation is the prevention of replication stress, which internally contradicts with the initial figures. The authors, should, therefore, find parameters that may allow exploring the relevance of findings in figure 1-4, working within the window of opportunity of 8 hours described in Figure 1. I admit it is tricky, but the current presentation of results is misleading. I suggest evaluating gamma H2AX accumulation (QIBC) combining 4-8 hrs Chk1 inhibition (low dose 30nM UCN01) with 1 hr CPT or perhaps 4 hours HU to determine if an alteration in the steady-state levels of Chk1 would prevent or enhance gammaH2AX accumulation.

Other points

1. Abstract; "The CHK1 protein kinase is essential to deal with replication stress (RS) and ensure genome integrity and cell survival, yet, how and why basal levels and activity of CHK1 are maintained under physiological, unstressed conditions is not known." The "why" has been extensively addressed, Chk1 loss causes augmentation of origin firing, replication stress, and genomic instability.
2. In the screening Cul1 depletion has a stronger effect on Chk1 level than DDB1. Could the CUL1 result be validated?
3. There is a discrepancy between the effect of the siRNAs for ETAA1 in Figure 4b and C. While the efficiency in Chk1 downregulation is A-B-C in Figure 4B, A is less efficient than B in Figure 4C (and it cannot be compared with C). Please explain the discrepancy.
4. Figure 4E; siDBB1 reverts only partially the effect of siETAA1 but fully prevents the effect of Chk1inhibition in figure 3D. Please discuss.
5. Figure S2G vs S2H, the drop in Chk1 levels seems to be faster than the drop in S296. Please discuss.
6. Figure S2L- which cells were used? If U2OS were not used, please show the result in U2OS.
7. Figure S4A and B. Despite the quantification results, the reduction in Chk1 levels after siETAA1 is not easy to observe. Can other exposures or blots be shown?
8. Figure S4F: the induction of Chk1 by CPT in control samples is not observed. In other cell lines, such induction is observed. How should such a difference be interpreted?

Minor points

1. Figure 2B: It seems that UCN.01-30nM causes an increase in EdU intensity in S phase. Could this be quantified?
2. For clarity, TOPBP1 should be identified in Figure 4A

We would like to thank all reviewers for having taken the time to read and evaluate our manuscript, and for insightful and constructive comments to improve our work further. In particular, we were very encouraged to read that our study was considered convincing and interesting for a broad audience of scientists working on various aspects of genome integrity, and we therefore revised our manuscript according to the reviewers' helpful suggestions. All new additions are detailed in our point-by-point response below.

Reviewer #1:

The authors have investigated Chk1 levels and importance by titrating Chk1 inhibitors to cells. They convincingly show that there is destabilization of Chk1 after Chk1 inhibition or depletion of upstream Chk1 regulators as ATR and ETAA1. Somewhat surprisingly, they find that this occurs throughout the cell cycle, and not only when Chk1 activity could be expected to be triggered during S-phase. They further propose that Chk1 activity regulating Chk1 levels could function as a safe-guard to maintain genome stability: if the checkpoint regulation is faulty so that a basal Chk1 activity is not maintained, it could eventually lead to loss of Chk1 and massive DNA damage in S phase.

That there is destabilization of Chk1 after Chk1 inhibition is clear. Also that there is destabilization after depletion of upstream regulators as ATR and ETAA1. Chk1 destabilization after checkpoint inhibition has been noted earlier, but the clear merit here is that it is shown to be de-coupled from DNA damage. It is also interesting that Chk1 is shown to have a basal activity throughout the cell cycle. There is not a lot of mechanistic insight into how stabilization occurs though, but considering the central role of Chk1 for replication stress I think the auto-stabilization idea could be interesting to a wide audience interested in genome integrity.

We were delighted to read that this reviewer found our main findings clear and convincing, and of interest for a wide audience. Indeed, we also envisage that due to the central role of CHK1 in the replication stress response the finding of CHK1 steady-state activity regulating its stability may have broader implications.

Major points

A main point is that there is a vicious cycle (or negative circuit in abstract), in which a checkpoint defect leading to low Chk1 activity would destabilize Chk1, leading to even lower Chk1 activity, ultimately leading to RS. The main experiment in support of this is that siRNA-mediated depletion of Chk1 only eventually leads to p21/gH2AX in S-phase. Although the concept is plausible and interesting - and certainly should be included - I don't agree the experiments show the existence of such a cycle beyond doubt and think the text should be adapted to indicate it is an idea put forward by the authors. To show a vicious cycle it would be necessary to somehow decouple it - for example by adding low levels of Chk1 inhibitor to cells expressing a mutant Chk1 that is not destabilized by reduced activity. Getting such an experiment right could be a major investment and not absolutely necessary to put forward the idea. I would

recommend to rather clearly indicate in the text that it is a concept proposed by the authors (mainly abstract, results, legend fig 5 - in discussion it's more balanced).

We agree that it would be great to be able to experimentally decouple CHK1 inhibition from its degradation by employing non-degradable CHK1 mutants. In an attempt to do so, we had performed mass spectrometry on immunoprecipitated endogenous CHK1 in presence or absence of CHK1i and identified several ubiquitylation sites, some of which overlapped with previously identified ubiquitylation sites by the Gygi lab (Kim et al. Mol Cell. 2011 44(2): 325-340). However, mutating individual lysines to alanine did not result in a clear stabilization of CHK1, probably due to ubiquitylation often being promiscuous at the target site level. Based on these inconclusive initial attempts, we agree with the reviewer that getting such experiments right would be a major investment that would go beyond the scope of this study. We therefore followed this reviewer's suggestion to clarify that the vicious cycle idea is a proposed concept.

It is argued that TopBP1 does not contribute to maintain Chk1 levels, but the data for that in S4H is not convincing.

We replaced this panel by a new Western Blot covering all four conditions of single and combined knockdown of ETAA1 and TOPBP1 (new Figure S2L). We further extended our analyses of the relative contributions of ETAA1 and TOPBP1 in the context of basal and replication stress-induced CHK1 activation, also inspired by comments from reviewers 2 and 3, and we provide the results in the new Figure panels S2M and S2O. Taken together, and consistent with our initial figure, we observe a dominant effect of ETAA1 on CHK1 steady-state activity and stability (Fig. S2L,M,O), and a milder contribution of TOPBP1 (which, on the other hand, has a strong effect in the context of acute CPT-induced replication stress, Fig. S2N,O). These new results consolidate our original conclusion that ETAA1 plays an important role for basal CHK1 activity and stability, but they also let us rephrase our conclusions on TOPBP1 (pages 10 and 14, and our model figure 5). We thank this reviewer and the other reviewers for highlighting this important point.

Chk1 levels from imaging are shown as violin plots rather than dot plots - which is fine, but should be complemented by raw data as it makes it difficult to assess cell cycle effects except in fig S2F. In that one it may seem as Chk1 is not destabilized in S-phase? (at 8h the S-phase cells have been through treatment in G1). A dot plot of Chk1 levels vs DNA should be included. Similarly, the RPE data is crucial to show that this is not restricted to cancer cells. The RPE data on Chk1 levels should be re-plotted to also show the levels throughout the cell cycle.

We completely agree with this point. We now provide a new Figure 1G, in which CHK1 staining was combined with EdU labeling in order to visualize CHK1 destabilization in a cell cycle resolved manner. The displayed single cell data clearly show that CHK1 levels decrease in all cell cycle phases, both at the 4h and at the 8h time-point. Additional data in Fig. S1H-N consistently demonstrate that

CHK1 levels decrease in all cell cycle phases, both in U-2 OS and non-transformed RPE cells. While we see the benefit of showing single cell raw data (as in the new example in main Fig. 1G), during the course of this study we also realized that in certain instances violin plots are easier to read and comprehend, which is why we suggest to provide the supplemental Fig. S1H-N as violin plots, with Fig. S1L and S1N separated by cell cycle phase.

We would like to thank this reviewer once again for his/her constructive and helpful suggestions.

Reviewer #2:

In the present manuscript, Michelena et al present their work on the identification of a positive feedback loop by which CHK1 activity sustains CHK1 protein levels. The work is based on defining conditions by which a mild CHK1 inhibition leads to lower CHK1 levels, without significant DNA damage (Figures 1 and 2). The authors later show that CHK1 degradation in this situation is in part mediated by DDB1-guided proteasomal activity (which was known in the context of DNA damage) and ETAA1.

My overall impression of this work is that while showing that CHK1 activity sustains CHK1 levels is of interest, at this point the manuscript is a bit premature for publication. Some of the figures are redundant (Fig. 1/2), and others insufficient to support the claim made (Fig. 5). Altogether, I do not believe that this MS fits the standards that one expects from JCB.

We appreciate the time and interest taken by this reviewer to read and evaluate our work, and we were glad to read that he/she considers our main findings on CHK1 activity sustaining its stability of interest. Based on this reviewer's suggestions we combined the first two figures into one, reworked the manuscript into a more condensed format, and, also based on suggestions by reviewer 3, extended the final figure on consequences of impaired CHK1 activity and stability.

Other comments/suggestions:

#1 Is the degradation of CHK1 observed upon CHK1 inhibition reverted by CDK1 inhibition? In other words, is this a consequence of a premature activation of the mitotic program, where many repair and replication factors are degraded?

While this is a valid concern, for the following reasons we can exclude that the observed CHK1 degradation is a consequence of premature activation of the mitotic program:

- 1) The observed CHK1 degradation occurs in all cell cycle phases (not just in late S, G2 or M), and the cell cycle profile does not show major changes in our experimental conditions (e.g. new Fig. 1G).

- 2) The observed CHK1 degradation is detected already after 30 minutes of CHK1i (Fig. S1I,J) when only very few cells would be expected to show premature activation of the mitotic program.
- 3) When monitoring pH3S10 as a marker of the activated mitotic program, we did not observe any changes upon 30 minutes of low dose CHK1i (Fig. R1A). In other words, conditions that result in CHK1 inactivation and destabilization (30 min CHK1i) do not result in measurable premature mitotic entry.
- 4) Upon 8 h of low dose CHK1i, cells are indeed driven into mitosis (but also here the effect is moderate with 7% mitotic cells compared to 4% in control conditions), and this is reverted by CDK1 inhibition (Fig. R1A). However, despite this rescue of premature mitotic activation, CHK1 activity and levels are not reverted by CDK1 inhibition (Fig. R1B).

Taken together, we conclude that the CHK1 destabilization reported in our manuscript is not related to premature activation of the mitotic program. Due to the more condensed format of our manuscript and associated space limitations we provide the CDK1 inhibitor data for the reviewers only. We would be happy to include them into the manuscript, however, upon recommendation by the reviewer.

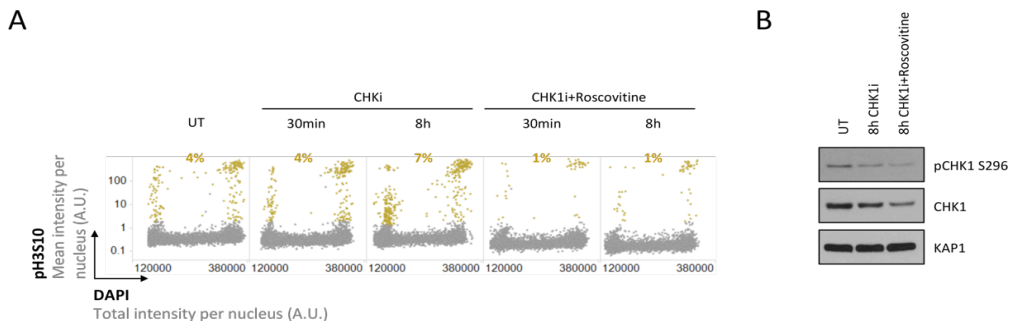


Fig. R1: Mitotic entry upon CHK1i. (A) U-2 OS cells were treated with CHK1i (30nM UCN-01) with or without roscovitine (20μM) for 30min or 8h as indicated, and stained for the mitotic marker phospho-H3 S10 (pH3S10) and DNA content. Percentages of cells in mitosis are indicated. (B) Western blot analysis of cells treated as in (A) for 8h.

#2 Since both MG132 and DDB1 depletion increase CHK1 levels even in the absence of CHK1 inhibition, it is difficult to prove that DDB1-guided proteasomal degradation is the mechanism that operates during CHK1 inhibition.

MG132 and DDB1 may indeed affect CHK1 levels also in absence of CHK1i. In our opinion, however, this does not argue against specific effects of proteasome inhibition or DDB1 loss in the context of CHK1i. In support, the relative changes upon DDB1 loss are greater in the CHK1i-treated samples compared to the untreated samples (Fig. 2D). Moreover, our new Fig. 2B shows that MG132 alone has a very moderate effect on CHK1 levels (in our experimental conditions), but almost completely rescues CHK1 levels upon CHK1i.

#3 The fact that TOPBP1 depletion does not affect CHK1 levels is difficult to understand. In contrast to ETAA1, TOPBP1 is essential at the cell level, and absolutely critical for ATR activity in all conditions. If the authors still want to make this case, they should perform solid genetic experiments to substantiate the dispensability of TOPBP1 in this phenomenon.

We are thankful for this important comment. We extended our analyses on ETAA1 and TOPBP1 functions (Fig. S2L-O), and indeed found that loss of TOPBP1 affects CHK1 levels and is thus not dispensable. However, the effects on CHK1 levels and stability are in all our experiments greater for ETAA1 depletion than for TOPBP1 depletion, while conversely the effects on CPT-induced CHK1 activation are greater for TOPBP1 than ETAA1 (Fig. S2L-O). In our revised model (Fig. 5) we thus attribute partly redundant roles to both proteins, consistent with synthetic lethality upon combined loss (e.g. Haahr et al. Nat Cell Biol. 2016 Nov;18(11):1196-1207), and also changed the text accordingly (pages 10 and 14).

#4 Figure 5 is a rather indirect way to make their point. It simply provides several evidences of genomic instability upon ETAA1 depletion or sustained CHK1 inactivation, which I would guess is rather expected.

We have reworked extensively the final figure of our manuscript, based on this reviewer's concern and suggestions by reviewer 3, and would kindly like to ask this reviewer to turn to our response to reviewer 3 to address this point.

#5 In general, I think the work provided here is not substantial for an Article. Most figures are rather scarce in data, or redundant with previous figures. I would suggest the authors to consider shorter and more condensed formats for the publication of this work.

Our revised manuscript is more condensed, and the results figures are comparably rich in data, typically containing between 7 and 16 individual figure panels. In several cases we complement our single cell imaging data with Western blot analysis of DNA damage markers or CHK1 protein levels, which could be perceived as redundant, but which can also make the main conclusions more robust. We hope that our revised and reformatted manuscript convincingly demonstrates our main conclusion that basal CHK1 activity safeguards its stability.

We would like to thank also this reviewer once again for raising important points and for helpful suggestions.

Reviewer #3:

The manuscript by Michelena J et al entitled "Basal CHK1 activity safeguards its stability to maintain intrinsic S-phase checkpoint functions" reports that the activation of Chk1 is required to maintain Chk1 steady-state levels in unchallenged cells. They also report that a reduction in Chk1 steady-state levels causes loss of the S-phase checkpoint, and compromised capability of cells to

respond to RS.

While the manuscript is potentially interesting, the biological implications of the protection of Chk1 stability by Chk1 activation in undamaged cells was not directly addressed. Another weakness of the manuscript is the limited tools used. Simple tools such as a kinase-dead mutant, antibodies against ATR-dependent phosphorylation of Chk1 and DNA combing technology will enrich the model proposed by the authors.

We are grateful to this reviewer for his/her interest and for making important suggestions for additional experiments to further corroborate and extend our work.

Major points

1) It is very well established that Chk1 loss causes alterations in DNA replication parameters (namely origin firing and DNA elongation rates) which in turn trigger gamma H2AX accumulation and double-strand break formation. This work demonstrated that moderate CHK1 inhibition affects the levels of Chk1 but not gamma H2AX and markers of double-strand break formation. It would be informative to establish whether replication parameters such as origin firing and DNA elongation are affected in such experimental settings.

We agree with this reviewer that providing replication fork measurements and origin activation data for our experimental conditions would be informative. We therefore performed DNA fiber experiments and scored fiber length (as a marker of replication fork speed) and origin initiation events. As rightly suspected by this reviewer, CHK1 inhibition in our experimental conditions resulted in an increase in origin initiation events and a corresponding decrease in fork elongation rates (new Fig. S3D,E). Importantly, when we combined CHK1 inhibition with CDC7 inhibition to block activation of new origins (Ahuja et al. Nat Commun. 2016 Feb 15;7:10660; Moiseeva et al. Nat Commun. 2017 Nov 9;8(1):1392; Mutreja et al. Cell Rep. 2018 Sep 4;24(10):2629-2642.e5.), we suppressed origin initiation and also rescued fork elongation rates, indicating that the main consequence of impaired CHK1 activity and reduced CHK1 stability is linked to unscheduled origin activation (see also point 4 below).

2) A Chk1 KD mutant should be used in this study. Basically, all experiments in Figure 3 should be performed with a Chk1 KD mutant. Accordingly to the model presented in this manuscript, the half-life of Chk1 KD should be shorter than the one of Chk1 wt because of increased proteolysis. Also, the use of Chk1 S345A and S317A mutants in half-life experiments and MG132-treatments may be informative.

The use of a CHK1 catalytically inactive mutant was an excellent suggestion. As CHK1 activity is essential for cell survival, precluding us from replacing the endogenous gene with mutant versions, we generated stable cell lines expressing CHK1 (either wild-type, or an S296A mutant, or a catalytically inactive D130A mutant (Chen et al. Cell. 2000 100(6): 681-692)) in a controlled, doxycyclin-inducible manner. All three versions were expressed at similar levels (new Fig. 2E), yet only the wild-type version showed S296 phosphorylation (new Fig. 2F).

We then performed protein stability measurements and consistently found that both the S296A mutant and the catalytically inactive D130A mutant were less stable compared to the wild-type counterpart (new Fig. 2G). These results are completely in line with our model and in our view represent an important new addition to our manuscript. We also generated cell lines expressing CHK1 S317A, S345A, S317A/S345A, or S317A/S345A/S296A. While all showed a moderately reduced CHK1 stability, only the triple mutant (containing the S296A mutation) showed completely abolished S296 phosphorylation, and this mutant had the strongest effect on CHK1 stability (Fig. R2). These results are overall consistent with our model, however we feel that a more extensive characterization of the single mutants would be required to fully understand the relative contribution of the individual phosphorylation sites on CHK1 stability. We would therefore prefer to provide these results for the reviewers only and focus on the S296A and the D130A mutants in the revised manuscript.

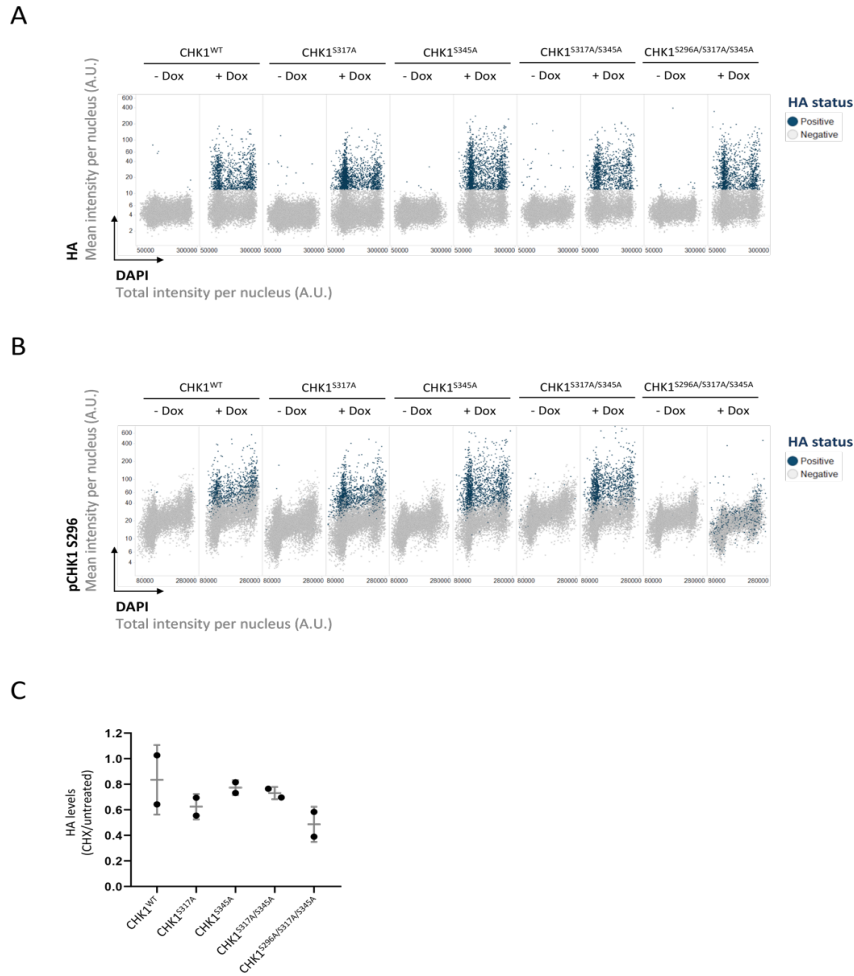


Fig. R2: Analysis of additional CHK1 mutants. (A) U-2 OS expressing either wild-type CHK1-HA or the indicated mutants upon Doxycycline (Dox) addition were identified based on the HA-signal. (B) The same cells as in (A) were stained for analyzed for pCHK1 S296. (C) Western blot quantifications of relative HA band intensities from two independent experiments of U-2 OS cells expressing either wild-type CHK1-HA or the indicated mutants, treated with CHX for 1h.

3) The levels of pCHK1S345 and pCHK1S317 should be monitored, especially when assessing the effect of ETAA1 and TOPBP1 depletion in the context of unchallenged proliferation or CPT treatment. Also, as Chk1 levels are regulated in all phases of the cell cycle, the evaluation of 296, 345 and 317 in G1, S, G2 would be very informative.

Also this was a valuable suggestion and we monitored the phosphorylation status of all three sites, S296, S317, S345, upon CPT treatment in siCon, siETAA1, and siTOPBP1 cells in our new Fig. S20. Consistent with our other data (Fig. S2), the effect of TOPBP1 loss is moderate on steady-state S296 phosphorylation, yet strong on CPT-induced S317 and S345 phosphorylation, while the effect of ETAA1 loss is moderate on the CPT-induced phosphorylations, yet dominant on steady-state S296 phosphorylation.

We would have loved to perform cell cycle resolved single cell analysis of pS317 and pS345, however in our hands only the pS296 antibody is specific enough in IF experiments to yield trustworthy results (Fig. R3). We hope that the reviewer agrees that this technical limitation precluded us from using such data in our revised manuscript.

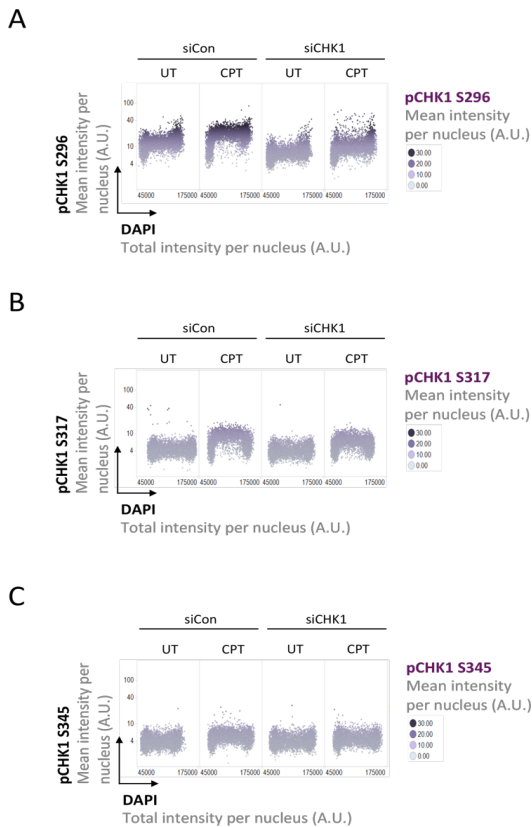


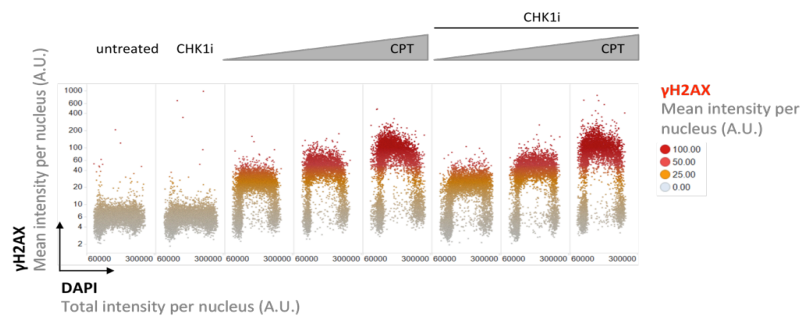
Fig. R3: QIBC analysis of phospho-CHK1 antibodies. (A) U-2 OS cells were transfected with siRNA against CHK1 for 24h, treated with CPT (1 μ M, 1h) and stained for pCHK1 S296 and DNA content. QIBC-derived pCHK1 S296 levels are shown as a function of cell cycle progression. (B) U-2 OS cells were treated as in (A) and stained for pCHK1 S317 and DNA content. (C) U-2 OS cells were treated as in (A) and stained for pCHK1 S345 and DNA content.

4) The assessment of the biological relevance of the findings in this manuscript is peculiar. In my opinion, Figure 5 builds upon a different aspect of Chk1 biology which is exploring the consequence of Chk1 loss (which is quite well documented). The settings used in Figure 5 (siChk1) are, so far, dissociated from the main subject of Figures 1-4. It is unclear if the authors assume that the levels of Chk1 observed after 24 hours of siChk1 (shown in Figure 5C) recapitulate the ones observed after 4 hours of low levels of Chk1 inhibitor (Figure 1). In any case, the link is confusing and so is the conclusion. Figure 1 indicates that, by controlling Chk1 activation, the levels of Chk1 can be modulated without affecting replication stress markers. In contrast, Figure 5 concludes that the relevance of maintenance of steady-state levels of Chk1 by Chk1 activation is the prevention of replication stress, which internally contradicts with the initial figures. The authors, should, therefore, find parameters that may allow exploring the relevance of findings in figure 1-4, working within the window of opportunity of 8 hours described in Figure 1. I admit it is tricky, but the current presentation of results is misleading. I suggest evaluating gamma H2AX accumulation (QIBC) combining 4-8 hrs Chk1 inhibition (low dose 30nM UCN01) with 1 hr CPT or perhaps 4 hours HU to determine if an alteration in the steady-state levels of CHK1 would prevent or enhance gammaH2AX accumulation.

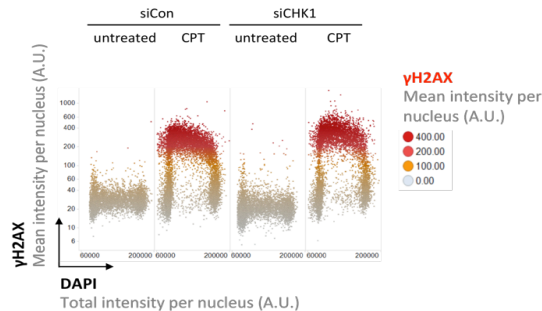
We are thankful for this comment and agree that the original data were indirect. We therefore followed the reviewer's suggestion to monitor the cellular response to acute replication stress (within a 4-8h time window) under controlled conditions of low dose CHK1i-induced CHK1 degradation. This was indeed technically challenging, but after several rounds of careful optimization we think we found conditions that allow conclusions about the consequences of CHK1 destabilization. In a series of experiments shown in revised Fig. 4 and S3, we show that CHK1 destabilization sensitizes cells to acute replication stress upon HU exposure. This sensitization occurs upon combined low dose CHK1i with HU (Fig. 4A-C), and also when cells are primed with CHK1i and then the CHK1i is washed out prior to the HU treatment (Fig. 4D-F). Under these conditions, CHK1 levels are lowered yet the remaining pool of CHK1 is efficiently activated by HU. Moreover, when we combined the CHK1i pulse to destabilize CHK1 with CDC7 inhibition to block new origin firing, this still led to a consistent sensitization to HU (Fig. 4G-I and S3D-G). In such conditions, CDC7i completely blocks the CHK1i-induced origin firing (Fig. S3E), and consequently rescues the reduced fork speed (Fig. S3D). The CHK1 that remains after CHK1i is activated by HU to the same extent as without CHK1i (Fig. S3H), thus showing that the washout of the CHK1i worked efficiently. We therefore conclude that lowering CHK1 levels impacts origin activity under conditions of replication stress. Consistent with this, when cells with lowered CHK1 were released into HU in presence of CDC7i, this rescued the hyper-sensitivity to HU (Fig. 4J,K). Collectively, our new results thus corroborate that lowered CHK1 levels, upon impaired steady-state CHK1 activity and the ensuing CHK1 degradation, are insufficient to properly maintain S-phase checkpoint functions, in agreement with previous studies demonstrating CHK1 haploinsufficiency (Lam et al., 2004; Boles et al., 2010). Once again, we would like to thank this reviewer for this insightful suggestion and believe that our revised final figure is now much more aligned with the rest of the manuscript.

In addition to the HU experiments, we also looked at CPT and found only a very moderate sensitization. This is presumably due to HU resulting in pronounced helicase-polymerase uncoupling and exposure of ssDNA, thereby heavily impacting origin firing, and CPT resulting in less exposure of ssDNA and global ssDNA-mediated origin suppression. Given that our new additions (incl. the CDC7i data) argue for deregulated origin firing as the main driving force behind CHK1 degradation-induced replication stress sensitivity (Fig. 4 and S3), we provide the CPT data for the reviewers only (Fig. R4). If recommended, and space permitted, we would be happy to discuss them also as counterpart to the HU data in our manuscript.

A



B



C

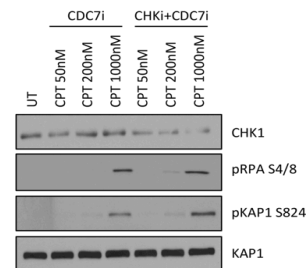


Fig. R4: Cellular response to CPT. (A) U-2 OS cells treated or not with low dose CHK1i (30nM) were exposed to increasing concentrations of CPT (50nM, 200nM, 1000nM) for 2h and stained for γ H2AX and DNA content. (B) U-2 OS cells were transfected with siRNA against CHK1 for 24h, treated with CPT (1 μ M, 1h) and stained for γ H2AX and DNA content. (C) Western blot analysis of U-2 OS cells treated with CDC7i in the presence or absence of low dose CHK1i for 4h and treated with CPT for 2h as indicated after washing out the inhibitors.

Other points

1. Abstract; "The CHK1 protein kinase is essential to deal with replication stress (RS) and ensure genome integrity and cell survival, yet, how and why basal levels and activity of CHK1 are maintained under physiological, unstressed conditions

is not known." The "why" has been extensively addressed, Chk1 loss causes augmentation of origin firing, replication stress, and genomic instability.

We agree and changed the abstract accordingly.

2. In the screening Cul1 depletion has a stronger effect on Chk1 level than DDB1. Could the CUL1 result be validated?

We could indeed validate the CUL1 result in individual assays. Due to space limitations we provide this result to the reviewers only (Fig. R5).

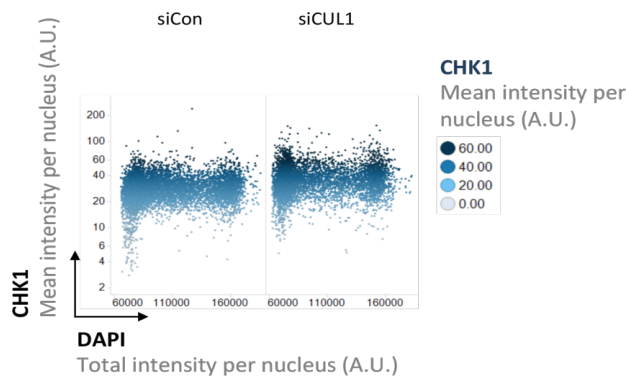


Fig. R5: Loss of CUL1 increases CHK1 levels. U-2 OS cells were transfected with siRNA against CUL1 and stained for CHK1 and DNA content.

3. There is a discrepancy between the effect of the siRNAs for ETAA1 in Figure 4b and C. While the efficiency in Chk1 downregulation is A-B-C in Figure 4B, A is less efficient than B in Figure 4C (and it cannot be compared with C). Please explain the discrepancy.

We believe that this discrepancy is most likely explained by technical aspects related to siRNA screens (in which knockdown efficiency cannot be directly controlled and in which well-to-well differences can cause additional variation) versus individual assays. We therefore repeated the individual siRNA transfections and ran the extracts on the same Western blot to allow better comparison and provide the result as new Fig. 3B. All three siRNAs against ETAA1 reduce pCHK1 S296A and CHK1 levels, and siRNA (B) has the strongest effect (as in the original Fig. 4C and in other experiments we had done with these siRNAs).

4. Figure 4E; siDBB1 reverts only partially the effect of siETAA1 but fully prevents the effect of Chk1inhibition in figure 3D. Please discuss.

We believe that this has to do with the timing of these experiments, as one is a simultaneous double depletion, whereas the other is a depletion of DDB1, which precedes a relatively short (4h) duration of CHK1i (in other words in the second scenario DDB1 is already depleted before CHK1 degradation is induced).

Extending the DDB1 knockdown (which would be required to achieve a similar situation in the context of ETAA1 depletion) led to significant cell stress, evidenced by high levels of γ H2AX (Fig. R6), making downstream analyses of CHK1 functions impossible. We thus believe that the partial versus full rescue is due to technical reasons and does not compromise our main conclusions.

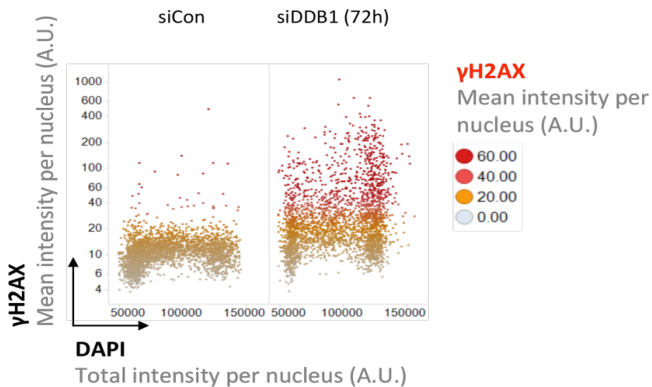
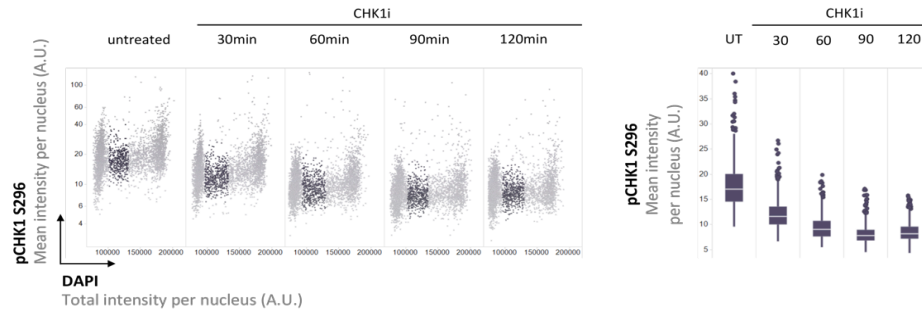


Fig. R6: DDB1 depletion for more than 48h is not well tolerated by cells. U-2 OS cells were transfected with siRNA against DDB1 for 72h and stained for γ H2AX and DNA content.

5. Figure S2G vs S2H, the drop in Chk1 levels seems to be faster than the drop in S296. Please discuss.

As these readouts are based on two different antibodies, one against CHK1 and one against pS296, the relative changes may not be comparable at such an absolute quantitative level. We provide an additional figure for the reviewers to show the cell cycle resolved single cell scatter plots of these experiments (Fig. R7), which for space reasons we did not include in the revised manuscript. There are mild differences between the profiles; when focusing the quantification on early S-phase cells, however, the relative drop in pCHK1 S296 is very comparable to the drop in CHK1 (to 68% at the 30 min time-point for pCHK1 S296, and to 69% for CHK1).

A



B

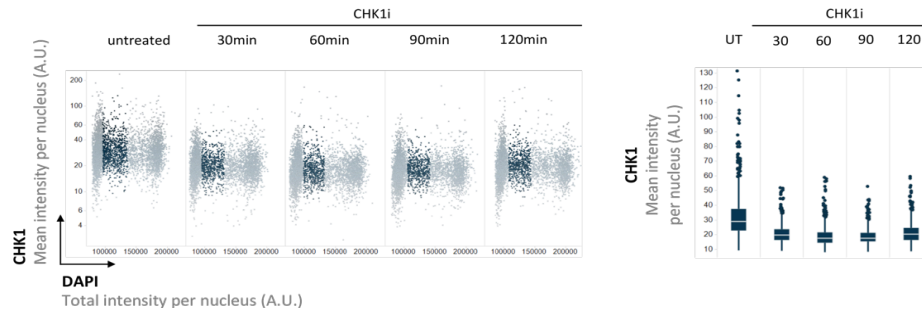


Fig. R7: Kinetics of loss of pCHK1 S296 and CHK1. (A) U-2 OS cells were treated with low dose CHK1i as indicated and stained for pCHK1 S296. QIBC-derived levels of pCHK1 S296 in the marked cell populations are shown as box plots. (B) U-2 OS cells were treated as in (A) and stained for CHK1. QIBC-derived levels of CHK1 in the marked cell populations are shown.

6. Figure S2L- which cells were used? If U2OS were not used, please show the result in U2OS.

This was indeed done in U-2 OS cells. We clarified this in the figure legend.

7. Figure S4A and B. Despite the quantification results, the reduction in Chk1 levels after siETAA1 is not easy to observe. Can other exposures or blots be shown?

We repeated these experiments and replaced the figures with the new Western blots (new Fig. S2E,F). The results are overall very similar to the previously shown ones, and the reduction in CHK1 levels after siETAA1 is easier to appreciate in the new replacement figures.

8. Figure S4F: the induction of Chk1 by CPT in control samples is not observed. In other cell lines, such induction is observed. How should such a difference be interpreted?

This is due to the fact that only the S-phase population responds to the CPT treatment and that RPE cells have a strong G1 peak and a relatively small S-phase population (Fig. R8). For space limitations and in order not to overload the manuscript we provide this for the reviewers only. It is nevertheless possible to appreciate the induction in the violin plots (Fig. S2J): While the median indeed only changes moderately (due to the high proportion of cells in G1), the upper part of the violin plot gets wider upon CPT treatment and this represents the S-phase population that responds to the treatment. If recommended, and space permitted, we would be happy to provide data from Fig. R8 also in the revised manuscript.

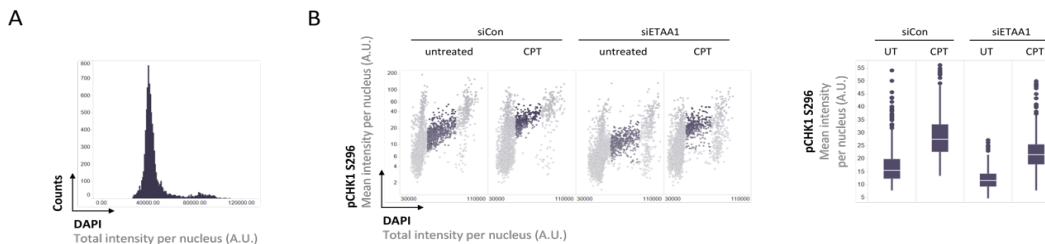


Fig. R8: S-phase analysis of RPE-1 cells. (A) One dimensional cell cycle profile of RPE-1 cells based on DAPI staining. (B) RPE-1 cells were transfected with siRNA for 48h, treated with camptothecin (CPT, 1 μ M) for 1h as indicated, and stained for pCHK1 S296. QIBC-derived levels of pCHK1 S296 are shown. On the right, box plots depict pCHK1 S296 levels from the same experiment focused on the marked cells in S-phase.

Minor points

1. Figure 2B: It seems that UCN.01-30nM causes an increase in EdU intensity in S phase. Could this be quantified?

This is correct, and probably due to excessive origin activation upon CHK1i (see new Fig. S3E). We quantified the increase in EdU and provide the data for the reviewers only (Fig. R9). If recommended, and space permitted, we would be happy to provide data from Fig. R9 also in the revised manuscript.

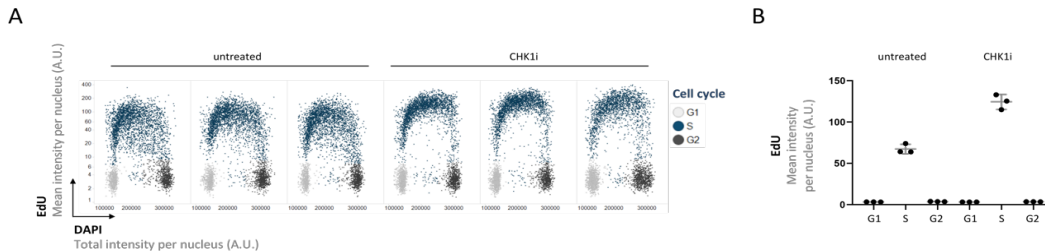


Fig. R9: EdU profiles upon low dose CHK1i. (A) U-2 OS cells were treated in triplicates with low dose CHK1i as indicated, pulse labeled with EdU (20min) and stained for EdU and DNA content. (B) QIBC-derived levels of EdU in (A) are depicted according to cell cycle position. Cell cycle staging was performed based on EdU and DAPI.

2. For clarity, TOPBP1 should be identified in Figure 4A

As suggested by this reviewer we marked TOPBP1 in this figure (now Fig. 3A).

We would like to thank also this reviewer for insightful and constructive comments and for his/her interest in our work, and are grateful for helpful suggestions that allowed us to improve our study further.

June 25, 2019

RE: JCB Manuscript #201902085R

Dr. Matthias Altmeyer
University of Zurich
Department of Molecular Mechanisms of Disease
Zurich 8057
Switzerland

Dear Dr. Altmeyer,

Thank you for submitting your revised manuscript entitled "Basal CHK1 activity safeguards its stability to maintain intrinsic S-phase checkpoint functions". You will see that both returning reviewers find that the revisions strengthened the work and they are now supportive of publication. One referee suggests incorporating to the manuscript some of the data provided to the reviewers' attention in the rebuttal and we agree with this change (we can accommodate a reasonable extension of the character count for Reports). While Reports are limited to 5 main and 3 supplementary figures, each figure can span up to one entire page as long as all panels fit on the page. We feel that there is space to include the additional data in the Report (in particular in the main figures). Please feel free to contact us with any questions about these changes. We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below) and pending changes to address Rev#3's comments.

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2) Figure formatting:

Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please add molecular weight with unit labels on the following panels: 1AB, 2ACDG, 3B, 4CFIK, S1ABP, S2AEFGHILMOP, S3G

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends.

Please indicate n/sample size/how many experiments the data are representative of: 1AB (quantifications), rest of figure 1, 2BEF, 3CD, figure 4, S1, S2BCDJKN, S3

4) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

- Please include the sequences for all siRNA oligos used in the study, including negative controls, if

those were made available to you from the manufacturer.

5) A summary paragraph of all supplemental material should appear at the end of the Materials and methods section.

- Please include ~1 sentence/supplementary item.

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in the Journal of Cell Biology.

Sincerely,

Agata Smogorzewska, MD, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD

Reviewer #1 (Comments to the Authors (Required)):

The authors have addressed my points and I recommend publication

Reviewer #3 (Comments to the Authors (Required)):

In this manuscript, the authors show that Chk1 levels are controlled in a manner that is de-coupled from DNA damage and depends on upstream Chk1 regulators as ATR and ETAA1. They also show that the basal levels of CHK1 are relevant to the extent of the response to DNA damaging agents (current Figure 4). I believe that the current version of the manuscript is very much improved. I also would like to mention that the authors did an important effort to cover most reviewers' comments. I personally believe that some of the data in the letter should be included in the manuscript. First, Fig. R4 is very informative and I think it adds to the description of the biological relevance of the findings described in the manuscript. Second, I believe that the effect of roscovitine on CHK1 levels (Figure R1) should definitely be included in the manuscript. Roscovitine treatment certainly reverts the defects in nascent DNA elongation and origin firing (plenty of manuscripts would support this claim), yet the levels of Chk1 are not altered by roscovitine (Fig. R1-Chk1i and Chk1i/Rosc lanes show equal levels of Chk1). Such result indicates that the control of Chk1 steady-state levels is uncoupled from both replication control (fiber results in Fig. S3) and DNA damage signals (gamma H2AX in Fig. 1). I do not really know if there is a timing effect (hours under roscovitine treatment) that precludes this conclusion but I believe that such W.B. is important.