EMBO reports

CHK1-CDC25A-CDK1 regulate cell cycle progression and protect genome integrity in early mouse embryos

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1st Editorial Decision **22nd Dec 2022**

Dear Dr. Drutovic,

Thank you for submitting your research manuscript for consideration by EMBO reports. It has now been seen by four experts in the field, and we have received the full set of their reports that is appended below.

As you will see, the referees acknowledge that the manuscript addresses an important question and that the findings are potentially interesting. However, they also identify some limitations and raise some technical concerns, and they provide a number of 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 usually recommend a revision within 3 months (March 21st). Please discuss with me the revision progress ahead of this time if you require more time to complete the revisions.

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We perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

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Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

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6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

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7) Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>).

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below (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.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

8) We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the new policy (<https://www.embopress.org/competing-interests>) and update your competing interests statement if necessary. Please name this section 'Disclosure and competing interests statement' and place it after the Acknowledgements section.

9) Figure legends and data quantification:
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.

See also the guidelines for figure legend preparation:
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- Please also include scale bars in all microscopy images.

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11) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at <https://www.embopress.org/page/journal/14693178/authorguide#referencesformat>.

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We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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

Yours sincerely,

Ioannis Papaioannou, PhD
Editor
EMBO reports

Referee #1:

In this manuscript, the authors study the functions of CHK1 in early mouse embryos. They report that CHK1 is responsible for a long G2 and also for preventing DNA damage as well as chromosome segregation errors. In the end, they speculate about the role of CHK1 in human fertility.
Overall, this manuscript addresses a very important question and the work is done very carefully (rare these days). Multiple approaches are used to confirm results including "rescue" experiments. This manuscript is nice to read even though is very detailed. The only "issue" is that besides the genetic models, a lot of chemical inhibitors are used, which we all know are not 100% specific. There are a few minor issues that would improve this manuscript.
Major issues:
1. On line 217/218, the authors imply that in the Chk1 mKO this is no issue with DNA replication and DSB in zygotes and on lines 232 in embryos. Is this compatible with the suggested role of CHK1 in S-phase?
2. Line 274: maybe not all readers will understand "In toto,..."
3. Fig.5B: is it possible to stain with gamma-H2X at the same time?
4. As every manuscript, a section describing the limitations would be beneficial. Especially discussing the issue of using "unspecific" chemical inhibitors.
5. Is there a way to actually measure the CDK1 activity (for example in Fig.3).

Referee #2:

Lucie et al. investigate the role of Chk1 in regulating the first two cell divisions of murine zygotes. Their approach is to use conditional gene deletion to deplete the maternal store of Chk1 protein and then to document the consequences for cell division using imaging in combination with inhibition/ augmentation of other key cell cycle components, such as CDC25A and CDK1, that are considered to be regulated by Chk1 based on extensive studies in somatic cells. They show that depletion of maternal Chk1 advances the onset of mitosis in both the first and second cell divisions after fertilization, with a more pronounced effect on the latter that results from a marked abbreviation of the G2 phase. Evidence is presented that these cell cycle alterations are attributable to mis-regulation of CDK1, most likely mediated primarily through loss of Chk1 control of CDC25A activity. Proper cell cycle timing is evidently critical for genomic stability during early embryogenesis, since Chk1 depleted embryos suffer from an increased frequency of multiple genomic aberrations including micronucleation and damaged and mis-segregated chromosomes. Based on these findings the authors propose that the Chk1-CDC25A-CDK1 pathway plays a critical role in protecting genomic integrity during early embryogenesis by ensuring the correct duration and timing of cell cycle phase

transitions, although the exact cause of the genomic damage that results from cell cycle dis-regulation remains unclear.

The experiments have been carefully executed and in the main correctly interpreted, however there are a number of issues that require some additional clarification/ explanation.

Major points

1) The segregation of the Chk1 mKO zygotes into two distinct phenotypic groups, designated Class I and Class II raises some important issues. Firstly, what is the basis of this distinction? Evidence is presented that Class II zygotes retain more maternal Chk1 protein expression than Class I (Fig. S1I). Two main possible explanations can be considered. A) That recombination of the floxed Chk1 allele did not occur during oogenesis in eggs giving rise to Class II zygotes. In essence this would mean that these zygotes would be essentially equivalent to WT, which in multiple assays they seem to be (eg Fig. 1E, Fig. 2F, Fig. 3A). More subtle differences from WT evident in other data might in this scenario be explained by differences in expression of the WT and floxed Chk1 alleles during oogenesis. Obviously this possibility can be easily ruled out by PCR to assess the recombination state of the floxed allele in Class II zygotes - has this been done? B) Assuming the Class II zygotes are recombined, an alternative explanation might be that the timing of flox-Chk1 recombination during embryogenesis varies leading to variable deposition of maternal Chk1 in eggs. If this were the case however one might expect to see substantial phenotypic heterogeneity in assays such as division time, yet in Fig. 1E, Fig. 2F, Fig. 3A the Class I and Class II populations are coherent with Class II essentially indistinguishable from WT. Some additional explanation of what is going on is required.

2) A related point: the Chk1 mKO population is divided into Class I and Class II in some datasets but not others. Presumably this is because for certain measured characteristics the population is behaving homogenously, however it is confusing, and there are instances, such as Fig. 3D, G, where one might expect to see Chk1 mKO Class I and Class II events, yet none are indicated. Unless these are in fact the events marked as mKO1 and mKO2, which are not really explained. Some further clarification would be helpful.

3) Section 3 "CHK1 kinase does not participate in replication checkpoint". Not convinced that this conclusion is supported by the data presented. Conventionally the replication checkpoint is demonstrated by blocking DNA replication using hydroxyurea (HU) or aphidicolin and thus arresting cells in S-phase. A functional replication checkpoint manifests as a block to mitotic entry that persists well beyond the time at which mitosis would otherwise have occurred had replication been completed. Many studies in somatic cells have established that Chk1 is essential for this response and that in the absence or inhibition of Chk1 cells will enter mitosis from S-phase with un-replicated DNA. The situation here however is quite different: essentially the data show that both WT and Chk1 mKO embryos complete S-phase and enter G2 with the same amount of DNA. Given that this is occurring in the absence of any external perturbation to inhibit DNA replication, it is not clear that that the replication checkpoint per se is actually being tested, particularly since there is no evidence that the checkpoint measures the quantity of genomic DNA. A more precise conclusion from this data would be that Chk1 is not essential in early embryonic divisions for completion of DNA replication, entry to G2, or subsequent mitosis in the proper order, albeit with altered timing. This has also been observed in at least some studies in somatic cells.

Minor points

1) The truncated Chk1 protein form detected in Chk1 mKO zygotes in Fig. S1E is evidently not detected after cell cycle progression as shown in Fig. S1I. Is this because this form is selectively lost with time, or a technical issue as the mKO signals are quite weak? Also, Fig. S1J seems to show that in most mice Class I zygotes predominate, yet in Figs. 1-3 Class I and Class II seem approximately equal.

2) Line 173-175: states "These changes in timing of cell division were due to loss of maternal CHK1 because microinjecting Chk1 mKO zygotes with Chk1-Egfp cRNA, but not Egfp cRNA, rescued the mutant phenotype (Fig. 1G). Yet Fig. 1G presents "Prop. zygote to blastocyst" which presumably means an endpoint measurement of percentage embryos reaching blastocyst stage rather than cell cycle transition timing?

3) Fig. 6F looks like data are missing for Chk1 WT + amanitin. This may simply be because these embryos are arrested at the two-cell stage and thus not "normal", but it is a little confusing.

4) Fig. S1C: genotype of female mice are denoted Chk1 Flox/ Zp3-Cre where they (compared to males Chk1 +/+) should be Chk1 flox/ flox; Zp3-Cre to avoid confusion.

5) Fig. 7G: Description of the model is minimal - presumably the red line is intended to denote CDC25A activity in the two genetic backgrounds but this is not stated.

6) The last sentences of the discussion refer to the importance of understanding cell cycle regulation during early embryogenesis in view of its possible relevance to human infertility. It would seem appropriate here to cite two recent studies that show that mutations in Chk1 form the basis of a heritable form of infertility in humans attributable to zygote arrest: Chen et al. 2021 PMID: 33948904, and Zhang et al. 2021 PMID: 33953335.

Referee #3:

The manuscript from Knoblochova et al. entitled "CHK1-CDC25A-CDK1 regulate cell cycle progression in early mouse embryos to protect genome integrity" reports the investigation of the roles of CHK1 and CDC25A in the first divisions of mouse embryos. The authors found that CHK1 inactivation affects the peculiar timing of the first and second cell cycles following fertilization. In particular, CHK1 KO accelerates the second division, leading to chromosome segregation defects and abortive development of blastocysts. Hence, this work provides some important information on the contribution of CHK1 during preimplantation development.

Altogether, I found that this manuscript is well written, most experiments are well designed and convincing with the corresponding controls. Nonetheless, some important points need to be addressed, as detailed below.

Main points:

1. My main concern is about the interpretation from the authors of the role of CDC25A in the control of mitotic entry of 2 to 4-cell stage embryos.
Indeed, using a fluorescent construct Cdc25a-Egfp, the authors found that CDC25A is rapidly degraded at 2-cell stage (Figure 3C, F), notably during G1 to S progression in a CHK1-dependent manner.
Because overexpression of CDC25A, following microinjection, accelerates the 2 to 4-cell division, it suggests that CDC25A degradation is required to slow-down the second cell cycle, possibly S phase progression according to previous work (for example: Sorensen Cancer cell 2003).
Conversely, data provided show that CDC25A do not reaccumulate during the several hours long G2 phase (Figure 3C, F). Hence, it seems unclear how CDC25A could control mitotic entry if not expressed.
Instead, accurate control of mitotic entry of 2 to 4-cell stage embryos could involve CDC25B or CDC25C that were not investigated in the present work. Both CDC25B and CDC25C are known targets of CHK1, inhibited by phosphorylation without degradation.
Can the authors provide additional data excluding a role of CDC25B and/or CDC25C in the control of mitotic entry, CHK1-dependent, from 2 to 4 cell stage?

2. The authors proposed that there is no DNA under-replication in CHK1 KO zygotes, based on measurements of DAPI staining. Can the authors provide data showing that this approach is sensitive enough? For example, partial inhibition of DNA replication showing lower DAPI staining 28h post-hCG (corresponding to the time of G2 phase in CHK1 WT zygotes, Fig S2A).

Minor points:
Lane 59: the sentence "mitotic G1 phase" is confusing and should be reformulated
Timing on Figure 2B is incorrect at the bottom right corner (58:20 not 34:30).
Lane 300: The sentence should be reformulated. CHK1 can mediates CDC25 phosphatases inactivation without degradation.

Referee #4:

In the submitted manuscript, Knoblochova et al describe the role of Chk1 in regulating cell cycle progression in early mouse embryos. Using a Chk1 knock-out mouse model, they revealed that zygotes lacking Chk1 divide sooner compared to Chk1 WT embryos. Notably, the authors observe two populations of embryos (class I and class II), with class II embryos having similar kinetics as WT embryos. The authors conclude that residual levels of Chk1 account for the absence of a phenotype. Using a modified Fucci sensor, the authors can show that class I Chk1 KO embryos have shortened G1, S and most prominently G2 phases suggesting that Chk1 is responsible for the long G2 phase in 2-cell embryos. Applying partial Cdk1 inhibition, Knoblochova et al could further show that premature Cdk1 activation accounts for the shortened G2 phase observed in Chk1 KO embryos. Analyses of Cdc25a-eGFP revealed an increase in Cdc25a-eGFP levels in Chk1 KO embryos. Loss of Cdc25a resulted in a significant delay in the second mitosis. Finally, the authors show that the long G2 phase in 2-cell embryos is essential for genome integrity.

The finding of Chk1 function in mammalian zygotes is interesting. The key finding - as mentioned in the abstract - is that CHK1 kinase regulates cell cycle progression in early mouse embryos by restraining Cdk1 activity due to Cdc25a degradation. Unfortunately, the manuscript is not very focused and therefore, the key finding is not very convincingly supported by the presented data. In general, the manuscript is written in a rather confusing manner which makes it difficult to read. Therefore, I recommend publication in a more specialized journal or the authors re-submit a revised version demonstrating more convincingly their key findings. Minor points:

- Line 68: it is mentioned that G2 phase is "virtually absent" in 2-cell embryos, whereas it is said to last 1-2h in line 60

- Lines 76-78: it is not clear if this statement just refers to mammals, but in early Xenopus embryos, work from the Ferrell lab has shown that the Wee1/Myt1 to Cdc25 ratio is critical for the difference in length of the first and second cell division (Tsai et al., 2014)
-
- Lines 111-112: in addition to the mentioned papers, in Xenopus it has been shown that adjustment of cell cycle length by CHK1 during the midblastula transisiton is mediated via degradation of Drf1 (Collart et al., 2017). This could also be mentioned in the discussion paragraph about the evolutionarily conserved role of Chk1.

- Fig. S1A: a possible truncated CHK1 version expressed in the KO embryos is mentioned. Is it known if this version has kinase activity?

- Fig. S1I and 1E: this reviewer is a bit surprised that the very small amount of residual CHK1 is sufficient to abolish the effect on cell division timing in Chk1 mKO classII 2- cell embryos? Is it possible to measure the Chk1 activity in the different embryos? It could also be interesting to test if the stability of Cdc25A-EGFP differs in classI and classII Chk1 mKO embryos.

- Fig. 1E and Fig. 5D: the classII embryos do not show altered timing of the second embryonic division (Fig. 1E). However, they show massive increases in segregation errors (Fig. 5D). What is the reason for these segregation errors?

- Fig. 1G: the figure legend says that the data are pooled from two experiments and that one of the two experiments contained the condition "Chk1 mKO + Chk1-Egfp cRNA" and the other experiment contained the condition "Chk1 mKO + Egfp cRNA", which implies that these two conditions were done just once. How can then a standard error be calculated for these two conditions (as shown in the graph)?

- S4E and S4F: in the text, it is mentioned that these experiments were conducted to investigate if increased Cdc25A levels cause premature mitotic entry. However, in both experiments there is no condition without overexpressed Cdc25A with which the timing in the case of overexpression could be compared

- Fig. 3C and 3D: the way the graphs are presented, it is impossible to deduce that the degradation of Cdc25A (before NEBD) is impaired in the Chk1 mKO embryos. In my opinion, it would be very helpful to also show a second graph with a much shorter time window on the x-axis, to see if Cdc25A is indeed more stable between the initial peak and the sudden decline at NEBD in the Chk1 mKO embryos, compared to the Chk1 WT embryos. If the lack of Cdc25A degradation is the reason for the observed phenotype of shorter G2 phase, then it should be possible to see a difference in the Cdc25 levels in this short time window, even without the partial Cdk1i in Fig. 3F and G.

- Fig. 3C and 3F: How do the authors explain the observation that the degradation of Cdc25A before NEBD is slowed down by the Cdk1i in the Chk1 WT embryos?

- Fig. 3: All conclusions from this figure depend on results with ectopic Cdc25A protein. Although not so easy to do with mouse oocytes, this figure would profit from an experiment showing that the levels of endogenous Cdc25A protein are reduced during the second division in WT embryos.

- Fig.4: the authors show that KO of Cdc25A delays cell division in the 1-cell and 2-cell embryos. One key prediction from this manuscript would be that in these embryos the depletion/inhibition of Chk1 should not result in acceleration of cell division, if it acts via Cdc25A destabilization. This should be tested.

- Fig. 6: to me, it is not really clear what the implications of the observation are that α-amanitin induces an arrest that depends on Chk1. Is this also related to Chk1´s impact on Cdc25A stability?

- Line 515: do the authors mean "lengthening"?

Dear Dr. Papaioannou and Reviewers,

Enclosed, please find the revision of our manuscript entitled “CHK1-CDC25A-CDK1 regulate cell cycle progression in early mouse embryos to protect genome integrity”, submitted for consideration for publication in EMBO Reports. We made our best effort to address the concerns raised in the initial review process; these changes are highlighted in yellow in the revised manuscript. What follows is a point-by-point response to the Reviewers’ concerns.

Sincerely yours,

David Drutovic

Referee #1:

In this manuscript, the authors study the functions of CHK1 in early mouse embryos.
They report that CHK1 is responsible for a long G2 and also for preventing DNA damage as well as chromosome segregation errors. In the end, they speculate about the role of CHK1 in human fertility.
Overall, this manuscript addresses a very important question and the work is done very carefully (rare these days). Multiple approaches are used to confirm results including “rescue” experiments. This manuscript is nice to read even though is very detailed. The only “issue” is that besides the genetic models, a lot of chemical inhibitors are used, which we all know are not 100% specific. There are a few minor issues that would improve this manuscript.

We are pleased that the Reviewer found our story interesting and addressed below the Reviewer’s comments.

Major issues:

1. On line 217/218, the authors imply that in the Chk1 mKO this is no issue with DNA replication and DSB in zygotes and on lines 232 in embryos. Is this compatible with the suggested role of CHK1 in S-phase?

Based on the DAPI staining, we concluded that *Chk1* mKO zygotes and *Chk1* mKO *class II* embryos do not show extensive DNA under-replication. In response to the suggestion from Reviewer 2 (see major point 3), we modified the conclusion regarding the role of CHK1 in the replication checkpoint. Because most studies of CHK1 in somatic cells are conducted using pharmacological inhibitors, our observation that CHK1 is not required to complete the main phase of DNA replication could be explained by the compensatory role of another kinase when *Chk1* is depleted. Furthermore, we elaborate on this issue below (see major point 3 from Reviewer 2).

2. Line 274: maybe not all readers will understand “In toto,...”

We apologize for using the expression “in toto”. We replaced the phrase with the word “altogether”.

3. Fig.5B: is it possible to stain with gamma-H2X at the same time?

Recent work analyzed the dynamics of γH2AX in living cells using fluorescently labeled antigen-binding fragments (Trakarnphornsombat & Kimura, 2023). Because of technical reasons, we cannot complete this analysis at this time. Such an experiment would require optimizing live-cell imaging conditions to detect γH2AX alone and with a chromatin marker

using WT zygotes. To our knowledge, such experiments have not been conducted in the past. We believe that immunofluorescence detection of γH2AX in fixed embryos showing elevated double-strand breaks in 2-cell stage (Fig EV2E and I) and 5-cell stage (Ruth et al., 2021) *Chk1* mKO embryos, as well as a known function of CHK1 in DNA damage response in somatic cells, provides a conclusive test of the requirement for CHK1 in DNA damage response in mouse embryos.

4. As every manuscript, a section describing the limitations would be beneficial. Especially discussing the issue of using “unspecific” chemical inhibitors.

We added a paragraph in the Discussion describing the limitation of the study. These details are found starting at line 523 and read:

Although CHK1 kinase does not participate in completing the main phase of DNA replication (**Fig EV2**), we find that it is essential to preserve genome integrity by maintaining a long G2 phase in 2-cell mouse embryos by restraining CDK1 activity due to CDC25A degradation (**Fig 7G**). This conclusion is based on a conditional gene knockout approach and pharmacological inhibitors, noting that it is formally possible that non-specific effects of the inhibitors could contribute to the observed phenotype. Minimizing this possibility is that RO-3306 treatment inhibits resumption of meiosis in mouse oocytes (**Fig EV3A**), thereby showing the efficiency of CDK1 inhibition by RO-3306. In addition, we previously showed that PD0166285 treatment rescues milrinone-induced prophase I arrest in mouse oocytes (Ferencova et al, 2022), indicating an effective inhibition of WEE/MYT kinases. Absence of CHK1 in mouse embryos phenocopied the effect of CHIR-124 on blastocyst development (**Fig EV1F**) and timing of the first embryonic division (**Appendix Fig S2A**) but not on the timing of the second embryonic division (**Appendix Fig S2B**), a difference likely caused by the lower specificity and/or efficiency of the inhibitor.

5. Is there a way to actually measure the CDK1 activity (for example in Fig.3).

In the study by Levasseur et al., 2019, the authors used a CDK1 FRET biosensor for analyzing cyclin B1:CDK1 activity in live mouse oocytes. Employing this approach would be extremely challenging for the following reasons. First, it will require optimizing long-term live-cell imaging conditions using WT oocytes and zygotes expressing this biosensor and validating the sensor for early mouse embryos. Second, our system provides the capability to scan four channels simultaneously. For FRET detection, we can use the three imaging channels defined as the Donor channel, the FRET channel, the Acceptor channel, and the fourth channel for transmitted light. We cannot scan two additional fluorescence channels in this configuration, as shown in Fig. 3, where chromosome segregation (H2B-mCHERRY) and CDC25A-EGFP destruction profile were analyzed. Note that we show that partial inhibition of CDK1 slows down the cell cycle as well as CDC25A degradation (Fig 3F and G), rescues M phase timing in 2-cell stage *Chk1* mKO embryos (Fig 3A), as well as genome integrity protection at 3-4 cell stage *Chk1* mKO embryos (Fig 7E and F). We believe that these results provide a conclusive test of premature CDK1 kinase activation when *Chk1* is depleted.

Referee #2:

Lucie et al. investigate the role of Chk1 in regulating the first two cell divisions of murine zygotes. Their approach is to use conditional gene deletion to deplete the maternal store of Chk1 protein and then to document the consequences for cell division using imaging in combination with inhibition/ augmentation of other key cell cycle components, such as CDC25A and CDK1, that are considered to be regulated by Chk1 based on extensive studies in somatic cells. They show that depletion of maternal Chk1 advances the onset of mitosis in both the first and second cell divisions after fertilization, with a more pronounced effect on

the latter that results from a marked abbreviation of the G2 phase. Evidence is presented that these cell cycle alterations are attributable to mis-regulation of CDK1, most likely mediated primarily through loss of Chk1 control of CDC25A activity. Proper cell cycle timing is evidently critical for genomic stability during early embryogenesis, since Chk1 depleted embryos suffer from an increased frequency of multiple genomic aberrations including micronucleation and damaged and mis-segregated chromosomes. Based on these findings the authors propose that the Chk1-CDC25A-CDK1 pathway plays a critical role in protecting genomic integrity during early embryogenesis by ensuring the correct duration and timing of cell cycle phase transitions, although the exact cause of the genomic damage that results from cell cycle dis-regulation remains unclear.

The experiments have been carefully executed and in the main correctly interpreted, however there are a number of issues that require some additional clarification/ explanation.

We appreciate the reviewers' recognition of our experimental work and valuable comments, which helped improve the quality of the manuscript.

Major points

1) The segregation of the Chk1 mKO zygotes into two distinct phenotypic groups, designated Class I and Class II raises some important issues. Firstly, what is the basis of this distinction? Evidence is presented that Class II zygotes retain more maternal Chk1 protein expression than Class I (Fig. S1I). Two main possible explanations can be considered. A) That recombination of the floxed Chk1 allele did not occur during oogenesis in eggs giving rise to Class II zygotes. In essence this would mean that these zygotes would be essentially equivalent to WT, which in multiple assays they seem to be (eg Fig. 1E, Fig. 2F, Fig. 3A). More subtle differences from WT evident in other data might in this scenario be explained by differences in expression of the WT and floxed Chk1 alleles during oogenesis. Obviously this possibility can be easily ruled out by PCR to assess the recombination state of the floxed allele in Class II zygotes - has this been done? B) Assuming the Class II zygotes are recombined, an alternative explanation might be that the timing of flox-Chk1 recombination during embryogenesis varies leading to variable deposition of maternal Chk1 in eggs. If this were the case however one might expect to see substantial phenotypic heterogeneity in assays such as division time, yet in Fig. 1E, Fig. 2F, Fig. 3A the Class I and Class II populations are coherent with Class II essentially indistinguishable from WT. Some additional explanation of what is going on is required.

In response to the Reviewer's concern, we used single-cell PCR analysis to ascertain the floxed allele's recombination state in *Chk1* mKO *class II* 2-cell stage embryos. These data are found in a new figure (Fig EV1J) with the following revised text:

To further investigate the distinction between *Chk1* mKO *class I* and *class II* embryos, we assed Cre-mediated DNA recombination status using a single-cell PCR to distinguish between non-recombined and recombined *Chk1* alleles (**Fig EV1J**). When the substrate for the PCR is the non-recombined allele, the expected size of the amplification product is a 2.2 kb fragment, whereas when the substrate is the recombined allele, it is a 0.4 kb fragment. We show that Cre-mediated recombination of the *Chk1* gene apparently occurs in all analyzed *Chk1* mKO *class I* and *class II* embryos, where only the 0.4 kb fragment was detected in each sample (**Fig EV1J**).

In this study, we used a mouse model where Cre recombinase is expressed using the zona pellucida-3 (*Zp3*) promoter to remove floxed exon 2 of *Chk1* in oocytes. *Zp3*-Cre is expressed in oocytes beginning at postnatal day 5 in primary follicles, whereas *Gdf9*-Cre expression begins at postnatal day 3 in primordial follicles (Wasson et al., 2016). Because *Zp3* promoter-driven expression begins in primary follicles, our model allows the

accumulation of some CHK1 protein in oocytes in primary follicles. We suspect that in some *Chk1* cKO oocytes that give rise to *Chk1* mKO *class II* embryos, the residual amount of CHK1 protein, in combination with its stability during meiotic maturation and early embryo development, is sufficient to maintain the long G2 phase in 2-cell embryos, but is not sufficient to regulate other processes, such as DNA damage response, genome integrity protection, and normal blastocyst development. This observation suggests that a low threshold of CHK1 activity is sufficient for maintaining a long G2 phase in 2-cell stage mouse embryos, confirming the critical role of the prolonged G2 phase in genome integrity protection.

2) A related point: the Chk1 mKO population is divided into Class I and Class II in some datasets but not others. Presumably this is because for certain measured characteristics the population is behaving homogenously, however it is confusing, and there are instances, such as Fig. 3D, G, where one might expect to see Chk1 mKO Class I and Class II events, yet none are indicated. Unless these are in fact the events marked as mKO1 and mKO2, which are not really explained. Some further clarification would be helpful.

Data presented in Fig EV3F show that after *Cdc25a-Egfp* cRNA microinjection, *Chk1* mKO *class I* and *class II* embryos were not observed, but only one population of embryos dividing significantly earlier than *Cdc25a*-microinjected *Chk1* WT embryos was described. We believe CDC25A-EGFP overexpression accelerates the second division in *Chk1* mKO *class II* embryos. This proposal is consistent with our previous finding that CDC25A overexpression induces premature mitosis in 2-cell stage WT embryos (Fig 4E). On the other hand, we also show that a portion of CDK1-inhibited and *Cdc25a*-microinjected *Chk1* mKO embryos showed either WT-like timing of mitotic entry and slow decrease of CDC25A-EGFP levels (referred as mKO1) or accelerated NEBD followed by CDC25A-EGFP dispersion into the cytoplasm (referred as mKO2). We believe that the *Chk1* mKO *class I* group is related to the mKO2 group, and the *Chk1* mKO *class II* group is related to the mKO1 group. Because we cannot compare phenotypes under two conditions - *Cdc25a*-microinjected vs. non-microinjected group - we prefer to use the names mKO1 and mKO2 in the manuscript.

3) Section 3 “CHK1 kinase does not participate in replication checkpoint”. Not convinced that this conclusion is supported by the data presented. Conventionally the replication checkpoint is demonstrated by blocking DNA replication using hydroxyurea (HU) or aphidicolin and thus arresting cells in S-phase. A functional replication checkpoint manifests as a block to mitotic entry that persists well beyond the time at which mitosis would otherwise have occurred had replication been completed. Many studies in somatic cells have established that Chk1 is essential for this response and that in the absence or inhibition of Chk1 cells will enter mitosis from S-phase with un-replicated DNA. The situation here however is quite different: essentially the data show that both WT and Chk1 mKO embryos complete S-phase and enter G2 with the same amount of DNA. Given that this is occurring in the absence of any external perturbation to inhibit DNA replication, it is not clear that that the replication checkpoint per se is actually being tested, particularly since there is no evidence that the checkpoint measures the quantity of genomic DNA. A more precise conclusion from this data would be that Chk1 is not essential in early embryonic divisions for completion of DNA replication, entry to G2, or subsequent mitosis in the proper order, albeit with altered timing. This has also been observed in at least some studies in somatic cells.

We agree with the points raised here, and we modified the title of this paragraph and the conclusion statement. The title of the section now reads:
CHK1 kinase does not participate in completing the main phase of DNA replication

The conclusion statement of the section now reads:

Our results suggest that CHK1 does not participate in completing the main phase of DNA replication and entry in the G2 phase and subsequent mitosis in early cleavage-stage mouse embryos because DNA content in *Chk1 mKO* was similar to *Chk1 WT* (**Fig EV2B and H**).

Our results show that CHK1 kinase does not participate in the completion of the “main “DNA replication. This observation is in accordance with a recent study by Zonderland et al. 2022. They showed that CHK1 was not necessary to complete the early phase of DNA replication. However, they showed that CHK1 was required to complete DNA replication in the G2 phase. They demonstrated that ATR/CHK1 regulates late S/G2-M progression. The entry into G2 is marked by a low threshold of origin firing activity (Zonderland et al., 2022), but some replication activity remains. We infer that CHK1 can drive premature mitosis only after entering the G2 program after completing the S-phase progression. This proposal agrees with Fig. 2, which shows that even *Chk1* mKO *class I* embryos completed the S-phase. In *Chk1*-depleted or CHK1-inhibited cells, premature mitosis entry from G2 may cause DNA under-replication. Regarding our results, we assumed that this amount of under-replicated DNA might be below our detection limit (Fig EV2B and H).

Minor points

1) The truncated Chk1 protein form detected in Chk1 mKO zygotes in Fig. S1E is evidently not detected after cell cycle progression as shown in Fig. S1I. Is this because this form is selectively lost with time, or a technical issue as the mKO signals are quite weak? Also, Fig. S1J seems to show that in most mice Class I zygotes predominate, yet in Figs. 1-3 Class I and Class II seem approximately equal.

At this point, we do not know if the truncated CHK1 protein is selectively lost with time or if the protein was not detected because of technical issues. Whereas we did not detect the signal of truncated CHK1 protein after increasing exposure time, we suppose that it is selectively lost with time. Nevertheless, we cannot exclude the possibility that a residual amount of this form is still present in the 4-cell and later stages.

Differences in the proportion of *Chk1* mKO *class I* embryos in individual mice could be caused by using mice with different genetic backgrounds, as stated in the Methods section.

2) Line 173-175: states “These changes in timing of cell division were due to loss of maternal CHK1 because microinjecting Chk1 mKO zygotes with Chk1-Egfp cRNA, but not Egfp cRNA, rescued the mutant phenotype (Fig. 1G). Yet Fig. 1G presents “Prop. zygote to blastocyst” which presumably means an endpoint measurement of percentage embryos reaching blastocyst stage rather than cell cycle transition timing?

We revised this sentence, which now reads:

Reduced blastocyst formation was due to loss of maternal CHK1 because microinjecting *Chk1* mKO zygotes with *Chk1-Egfp* cRNA, but not *Egfp* cRNA, rescued the mutant phenotype (Fig 1G).

3) Fig. 6F looks like data are missing for Chk1 WT + amanitin. This may simply be because these embryos are arrested at the two-cell stage and thus not “normal”, but it is a little confusing.

We have added lines to the Figure legend to clarify that all α-amanitin-treated *Chk1* WT embryos were arrested in the 2-cell stage.

4) Fig. S1C: genotype of female mice are denoted Chk1 Flox/ Zp3-Cre where they (compared to males Chk1 +/+) should be Chk1 flox/ flox; Zp3-Cre to avoid confusion.

We apologize for the mistake, and we modified the figure legend.

5) Fig. 7G: Description of the model is minimal - presumably the red line is intended to denote CDC25A activity in the two genetic backgrounds but this is not stated.

We modified the paragraph to clarify the model. The text now reads:

In wild-type mouse embryos, active CHK1 (shown in purple) restrains CDK1 activity (gray) by promoting CDC25A degradation (dashed magenta line) to regulate cell cycle progression and protect genome integrity in 2-cell embryos. CDK1 is activated (black) by its newly transcribed activators after genome activation. Absence of CHK1 (crossed) causes premature CDK1 kinase activation (black) due to stabilization of CDC25A phosphatase (solid magenta line), which leads to premature mitosis and chromosome fragmentation. The magenta curve represents the amount of CDC25A protein in individual cell cycle phases in 2-cell stage wild-type and *Chk1* mKO embryos.

6) The last sentences of the Discussion refer to the importance of understanding cell cycle regulation during early embryogenesis in view of its possible relevance to human infertility. It would seem appropriate here to cite two recent studies that show that mutations in Chk1 form the basis of a heritable form of infertility in humans attributable to zygote arrest: Chen et al. 2021 PMID: 33948904, and Zhang et al. 2021 PMID: 33953335.

We apologize for the omission and have added the references.

Referee #3:

The manuscript from Knoblochova et al. entitled “CHK1-CDC25A-CDK1 regulate cell cycle progression in early mouse embryos to protect genome integrity” reports the investigation of the roles of CHK1 and CDC25A in the first divisions of mouse embryos.
The authors found that CHK1 inactivation affects the peculiar timing of the first and second cell cycles following fertilization. In particular, CHK1 KO accelerates the second division, leading to chromosome segregation defects and abortive development of blastocysts.
Hence, this work provides some important information on the contribution of CHK1 during preimplantation development.

Altogether, I found that this manuscript is well written, most experiments are well designed and convincing with the corresponding controls. Nonetheless, some important points need to be addressed, as detailed below.

We are pleased that the Reviewer found our manuscript well-written and addressed below the Reviewer’s comments.

Main points:

1. My main concern is about the interpretation from the authors of the role of CDC25A in the control of mitotic entry of 2 to 4-cell stage embryos.
Indeed, using a fluorescent construct Cdc25a-Egfp, the authors found that CDC25A is rapidly degraded at 2-cell stage (Figure 3C, F), notably during G1 to S progression in a CHK1-dependent manner.
Because overexpression of CDC25A, following microinjection, accelerates the 2 to 4-cell division, it suggests that CDC25A degradation is required to slow-down the second cell cycle, possibly S phase progression according to previous work (for example: Sorensen Cancer cell 2003).
Conversely, data provided show that CDC25A do not reaccumulate during the several hours

long G2 phase (Figure 3C, F). Hence, it seems unclear how CDC25A could control mitotic entry if not expressed.
Instead, accurate control of mitotic entry of 2 to 4-cell stage embryos could involve CDC25B or CDC25C that were not investigated in the present work. Both CDC25B and CDC25C are known targets of CHK1, inhibited by phosphorylation without degradation.
Can the authors provide additional data excluding a role of CDC25B and/or CDC25C in the control of mitotic entry, CHK1-dependent, from 2 to 4 cell stage?

Although we do not know the exact timing of the G1/S transition, our data suggest that CDC25A-EGFP accumulates during the G1 phase, peaks at G1/S, is degraded during the S-phase, and remains absent during the G2 phase (comparing **Fig 2C** and **Fig 3C**). The CDC25A-EGFP high level at the G1/S boundary suggests an utmost importance CDC25A role in initiating the S-phase start, as shown in (Vigo et al, 1999).

As noted by the Reviewer, we do not generally see that the CDC25A-EGFP signal increases before mitosis. Consistent with CDC25A regulating mitosis entry (Mailand et al, 2002) in human somatic cells, there are some individual embryos where there is an indication of signal rise before NEBD (abrupt signal drop indicated by asterisks) (**Fig 3C and 4G**). However, we propose that CDC25A regulation in mouse 2-cell stage embryos differs from that in somatic cells and that CHK1-CDC25A-CDK1 regulation was adapted in a developmental manner to prolong the G2 phase needed for genome activation.

We suggest that in 2-cell stage embryos, CDC25A is degraded during the S-phase. Under normal circumstances, it can be transcribed or translated prior to mitosis. However, 2-cell stage mouse embryos did not show any *Cdc25a* mRNA (**Fig 4A**), and their transcription has not yet been fully established. Therefore, we suggest that the long G2 phase is regulated exactly by the absence of *Cdc25a*; that is, *Cdc25a* absence results in G2 phase prolongation needed for genome activation.

Furthermore, mitotic entry occurs only after the transcription of a sufficient amount of *Cdk1* and CDK1 activators, such as (*Ccna2* and *Cdc25c*) (Abe et al, 2018, Park et al, 2013, Zeng et al, 2004). Analysis of the data from Zeng et al. (2004) showed that mRNA levels of *Cdk1* and *Cdc25c* significantly increased from the zygote to the 2-cell stage, whereas *Cdc25a* levels decreased after fertilization until the major genome activation at the 2-cell stage and *Cdc25b* levels remained unchanged. In addition, inhibition of transcription by α-amanitin (**Fig 6**) resulted in 2-cell stage arrest in wild-type but not *Chk1 mKO* embryos. In addition, CHK1 overexpression caused G2/M arrest and low CDC25A levels in *Xenopus* embryos. However, CDC25C expression remains unaffected (Hartley et al, 1996, Petrus et al, 2004). In contrast, CHK1 inhibition causes the upregulation of CDC25B in *Xenopus* (Lee et al., 2001).

In response to the Reviewer's concern and the suggestion from Reviewer 4, we confirm the hypothesis that CHK1 inhibition does not result in accelerating cell division in *Cdc25a* mKO embryos. We conducted bright-field time-lapse imaging using light-sheet microscopy and assessed the timing of the first and second mitosis in WT, *Cdc25a* mKO, CHIR-124-treated WT (CHK1 inhibition), and CHIR-124-treated *Cdc25a* mKO embryos. In a new Appendix Fig. S3, we show that CHK1 inhibition can accelerate the zygote to 2-cell division of WT but not *Cdc25a* mKO zygotes. We reason that the ability of CHIR-124 to accelerate the first mitosis in WT zygotes without affecting the 2-cell stage entry in *Cdc25a* mKO confirms that CHK1 acts via CDC25A destabilization in mouse embryos.

These observations suggest that CDC25A is the major CDC25 phosphatase during early embryonic development, and its stabilization explains the premature mitotic entry in Chk1 mKO embryos. Because CHK1 is known to regulate both CDC25B and CDC25C, we cannot fully exclude their role, especially that of CDC25B phosphatase.

2. The authors proposed that there is no DNA under-replication in CHK1 KO zygotes, based on measurements of DAPI staining. Can the authors provide data showing that this approach is sensitive enough? For example, partial inhibition of DNA replication showing lower DAPI staining 28h post-hCG (corresponding to the time of G2 phase in CHK1 WT zygotes, Fig S2A).

DAPI staining is not sensitive enough to detect the under-replication of a small fraction of the genome, e.g. if 95% of the DNA is replicated. Based on the DAPI staining, we conclude that extensive DNA under-replication, i.e., under-replication of a larger fraction of the genome, is not detected in *Chk1* mKO zygotes (Fig EV2B) and *Chk1* mKO *class II* embryos (Fig EV2H).

Minor points:
Lane 59: the sentence “mitotic G1 phase” is confusing and should be reformulated

We modified the sentence. Text now reads:

In contrast to the zygotic G1 phase, which lasts about 5-12 h, dependent on the genetic background, 2-cell stage embryos spend only 1-2 h in the G1 phase.

Timing on Figure 2B is incorrect at the bottom right corner (58:20 not 34:30).

This typo has been corrected.

Lane 300: The sentence should be reformulated. CHK1 can mediates CDC25 phosphatases inactivation without degradation.

We modified the sentence. Text now reads:

CHK1 disrupts the action of all CDC25 phosphatases through inhibitory phosphorylation, as in the case of CDC25B and CDC25C, but most importantly, through degradation, as in the case of CDC25A.

Referee #4:

In the submitted manuscript, Knoblochova et al describe the role of Chk1 in regulating cell cycle progression in early mouse embryos. Using a Chk1 knock-out mouse model, they revealed that zygotes lacking Chk1 divide sooner compared to Chk1 WT embryos. Notably, the authors observe two populations of embryos (class I and class II), with class II embryos having similar kinetics as WT embryos. The authors conclude that residual levels of Chk1 account for the absence of a phenotype. Using a modified Fucci sensor, the authors can show that class I Chk1 KO embryos have shortened G1, S and most prominently G2 phases suggesting that Chk1 is responsible for the long G2 phase in 2-cell embryos. Applying partial Cdk1 inhibition, Knoblochova et al could further show that premature Cdk1 activation accounts for the shortened G2 phase observed in Chk1 KO embryos. Analyses of Cdc25a-eGFP revealed an increase in Cdc25a-eGFP levels in Chk1 KO embryos. Loss of Cdc25a resulted in a significant delay in the second mitosis. Finally, the authors show that the long G2 phase in 2-cell embryos is essential for genome integrity.

The finding of Chk1 function in mammalian zygotes is interesting. The key finding - as mentioned in the abstract - is that CHK1 kinase regulates cell cycle progression in early mouse embryos by restraining Cdk1 activity due to Cdc25a degradation. Unfortunately, the manuscript is not very focused and therefore, the key finding is not very convincingly supported by the presented data. In general, the manuscript is written in a rather confusing manner which makes it difficult to read. Therefore, I recommend publication in a more

specialized journal or the authors re-submit a revised version demonstrating more convincingly their key findings. Minor points:

We are pleased that the Reviewer found our story interesting and addressed below the Reviewer's comments.

- Line 68: it is mentioned that G2 phase is "virtually absent" in 2-cell embryos, whereas it is said to last 1-2h in line 60

We modified the sentence. The text now reads:

The second division is characterized by a very short G1 phase (approximately 1-2 h) and a dramatic lengthening of the G2/M transition (approximately 12-16 h) compared to 1-8 h for G2/M in the first division.

- Lines 76-78: it is not clear if this statement just refers to mammals, but in early Xenopus embryos, work from the Ferrell lab has shown that the Wee1/Myt1 to Cdc25 ratio is critical for the difference in length of the first and second cell division (Tsai et al., 2014)

As the statement refers to mammals, we modified the sentence. The text now reads:

Mechanisms underlying regulation of cell cycle duration in the initial divisions of early mammalian development, in particular the transition from a long G1 and short G2 in zygotes to a short G1 long G2 in 2-cell embryos, remain largely unknown.

- Lines 111-112: in addition to the mentioned papers, in Xenopus it has been shown that adjustment of cell cycle length by CHK1 during the midblastula transisiton is mediated via degradation of Drf1 (Collart et al., 2017). This could also be mentioned in the discussion paragraph about the evolutionarily conserved role of Chk1.

We apologize for the omission and have added both references.

- Fig. S1A: a possible truncated CHK1 version expressed in the KO embryos is mentioned. Is it known if this version has kinase activity?

In *Chk1* mKO mice, *Zp3*-Cre was used to excise exon 2 of the maternal *Chk1* allele during oocyte growth (Lewandowski et al, 1997; Lam et al, 2004). Exon 2 contains the initiating translational start codon and N-terminal lobes, including Lys38, which forms an invariant ion pair with Glu55 in the ATPase domain. CHK1 kinase function is disrupted in this mutant (Chen et al. 2000).

- Fig. S1I and 1E: this Reviewer is a bit surprised that the very small amount of residual CHK1 is sufficient to abolish the effect on cell division timing in Chk1 mKO classII 2- cell embryos? Is it possible to measure the Chk1 activity in the different embryos? It could also be interesting to test if the stability of Cdc25A-EGFP differs in classI and classII Chk1 mKO embryos.

In the study by Lebrec et al., 2022, the authors used a CHK1 FRET biosensor for analyzing CHK1 dynamics in somatic cells. As described in response #5 to Referee 1, to our knowledge, such an assay has not been conducted in mouse oocytes or zygotes. Such experiments will require optimizing long-term live-cell imaging conditions using WT oocytes and zygotes expressing this biosensor and validating the sensor for early mouse embryos. Note that *Chk1 mKO class II* embryos had a higher amount of CHK1 compared to *Chk1 mKO class I* embryos. This observation suggests that a low threshold of CHK1 activity is

sufficient for maintaining a long G2 phase in 2-cell mouse embryos, confirming the critical role of the prolonged G2 phase in genome integrity protection.

We agree that testing the stability of CDC25A-EGFP in *Chk1* mKO *class I* and *class II* embryos is interesting. See response #2 in major points to Reviewer 2.

- Fig. 1E and Fig. 5D: the classII embryos do not show altered timing of the second embryonic division (Fig. 1E). However, they show massive increases in segregation errors (Fig. 5D). What is the reason for these segregation errors?

Chk1 mKO *class II* embryos exhibit increased DNA damage (Fig EV2I) and a shorter G1 and S phase (Fig 2D and E). It is well known that anaphase bridges, frequently observed in *Chk1* mKO *class II* embryos (Fig EV4C), can arise from chromosome fusion caused by DNA damage repair. However, we cannot exclude another possibility, such as segregation errors caused by cell-cycle deregulation or bipolar spindle defects. We believe that determining the causes of segregation errors in *Chk1* mKO *class II* embryos is beyond the scope of this study.

- Fig. 1G: the figure legend says that the data are pooled from two experiments and that one of the two experiments contained the condition “Chk1 mKO + Chk1-Egfp cRNA” and the other experiment contained the condition “Chk1 mKO + Egfp cRNA”, which implies that these two conditions were done just once. How can then a standard error be calculated for these two conditions (as shown in the graph)?

These two mentioned conditions were indeed done once. We computed the standard error as the standard error of proportion, which was computed from the number of embryos used. The number of used embryos is, in both cases, 24.

- S4E and S4F: in the text, it is mentioned that these experiments were conducted to investigate if increased Cdc25A levels cause premature mitotic entry. However, in both experiments there is no condition without overexpressed Cdc25A with which the timing in the case of overexpression could be compared

The data in Fig. EV3E and F were compared with the data presented in Fig. EV4A and B, which show the timing of first and second mitosis in *Chk1* WT and *Chk1* mKO embryos microinjected with *H2b-mCherry* cRNA.

- Fig. 3C and 3D: the way the graphs are presented, it is impossible to deduce that the degradation of Cdc25A (before NEBD) is impaired in the Chk1 mKO embryos. In my opinion, it would be very helpful to also show a second graph with a much shorter time window on the x-axis, to see if Cdc25A is indeed more stable between the initial peak and the sudden decline at NEBD in the Chk1 mKO embryos, compared to the Chk1 WT embryos. If the lack of Cdc25A degradation is the reason for the observed phenotype of shorter G2 phase, then it should be possible to see a difference in the Cdc25 levels in this short time window, even without the partial Cdk1i in Fig. 3F and G.,

We agree that viewing the degradation of CDC25A in the *Chk1* mKO embryos is difficult. We have added a new figure (Appendix Fig S2) with a shorter time window on the x-axis.

- Fig. 3C and 3F: How do the authors explain the observation that the degradation of Cdc25A before NEBD is slowed down by the Cdk1i in the Chk1 WT embryos?

Decreased CDK1 activity caused by treatment with CDK1 inhibitor slows the cell cycle, which in turn slowed down CDC25A degradation.

- Fig. 3: All conclusions from this figure depend on results with ectopic Cdc25A protein. Although not so easy to do with mouse oocytes, this figure would profit from an experiment showing that the levels of endogenous Cdc25A protein are reduced during the second division in WT embryos.

We used a commercially available DCS-122 antibody (Santa Cruz, sc-65503) for immunoblot detection of CDC25A. This antibody detects three bands in the expected size range of 70-80 kDa in WT but not *Cdc25a* mKO zygotes (data not shown). However, the detection of CDC25A requires 300-400 zygotes per sample. Conducting such experiments will require collecting thousands of 2-cell stage WT embryos. To comply with our Institute's mandate to minimize the number of animals used in research, we decided to optimize live-cell light-sheet imaging conditions and measured CDC25A-EGFP intensity in the 2-cell embryos with 10-min resolution. We believe that this analysis clearly shows the destruction of CDC25A regulated by CHK kinase.

Fig.4: the authors show that KO of Cdc25A delays cell division in the 1-cell and 2-cell embryos. One key prediction from this manuscript would be that in these embryos the depletion/inhibition of Chk1 should not result in acceleration of cell division, if it acts via Cdc25A destabilization. This should be tested.

We appreciate this suggestion to confirm the hypothesis that CHK1 inhibition does not accelerate cell division in *Cdc25a* mKO embryos. We conducted bright-field time-lapse imaging using light-sheet microscopy and assessed the timing of first and second mitosis in WT, *Cdc25a* mKO, CHIR-124-treated WT, and CHIR-124-treated *Cdc25a* mKO embryos. In a new Appendix Fig S3, we show that CHK1 inhibition can accelerate zygote to 2-cell division of WT zygotes but not *Cdc25a* mKO zygotes. We reason that the ability of CHIR-124 to accelerate first mitosis in WT zygotes without affecting 2-cell stage entry in *Cdc25a* mKO confirms that CHK1 acts via CDC25A destabilization in mouse embryos. These data are found in a new figure (Appendix Fig S3) with the following revised text:

Having confirmed that CHK1 regulates entry to mitosis by controlling CDC25A degradation in mouse embryos (**Fig 3D and G**), we were interested in testing whether CHK1 inhibition affects the timing of mitosis in *Cdc25a* mKO embryos. To do so, we isolated zygotes from WT and *Cdc25a* mKO mice and monitored the blastocyst development in the presence of 250 nM CHIR-124 using bright-field time-lapse imaging. Although the CHK1 inhibition slightly but significantly accelerated the first division in WT embryos, the timing of the first mitosis was not affected in *Cdc25a* mKO (**Appendix Fig. S3A**). As confirmed above (**Fig 4B, C, and E**), *Cdc25a mKO* embryos showed a delay in the first and second mitosis. However, treatment with CHIR-124 does not accelerate this timing of second mitosis in WT, as well as *Cdc25a* mKO embryos (**Appendix Fig. S3B**).

- Fig. 6: to me, it is not really clear what the implications of the observation are that α-amanitin induces an arrest that depends on Chk1. Is this also related to Chk1's impact on Cdc25A stability?

This experiment confirms our hypothesis that CHK1 inhibits the second mitosis entry until the transcription of a sufficient amount of *Cdk1* and CDK1 activators (*Ccna2*, *Cdc25c*) (Abe et al. 2018, Park et al. 2013, Zeng et al. 2004). Transcription of these CDK1 activators is required because of the absence of endogenous *Cdc25a* mRNA (Fig 4A) and possibly the absence of CDC25A protein (Fig 3C).

- Line 515: do the authors mean "lengthening"?

We apologize for this mistake and changed "shortening "to "lengthening ".

Dear Dr. Drutovic,

Thank you for the submission of your revised manuscript to EMBO reports and for your patience during peer review. We have now received the full set of reports from the four referees that were asked to re-evaluate your study. Please find their comments below.

As you will see, all referees find the revised version significantly improved, and they now recommend publication in EMBO reports. There is only one minor comment of referee #3, which I need you to address in a revised version of your manuscript.

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

- I took the liberty to edit slightly the title and the abstract. Please review my suggestions below and incorporate them into your revised manuscript if you agree with them, or feel free to improve them further, if you like:

Suggested title:
CHK1-CDC25A-CDK1 regulate cell cycle progression and protect genome integrity in early mouse embryos

Suggested abstract:
After fertilization, remodeling of the oocyte and sperm genomes is essential to convert these highly differentiated and transcriptionally quiescent cells into early cleavage stage blastomeres that are transcriptionally active and totipotent. This developmental transition is accompanied by cell cycle adaptation, such as lengthening or shortening of the gap phases G1 and G2. However, regulation of these cell cycle changes is poorly understood, especially in mammals. Checkpoint kinase 1 (CHK1) is a protein kinase that regulates cell cycle progression in somatic cells. Here, we show that CHK1 regulates cell cycle progression in early mouse embryos by restraining CDK1 kinase activity due to CDC25A phosphatase degradation. CHK1 kinase also ensures the long G2 phase needed for genome activation and reprogramming gene expression in 2-cell stage mouse embryos. Finally, Chk1 depletion leads to DNA damage and chromosome segregation errors that result in aneuploidy and infertility.

- The author contributions statement should be removed from the manuscript file. Instead, we now use CRediT to specify the contributions of each author in the journal submission system. Please use the free text box to provide more detailed descriptions. See also our guide to authors:
<https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>.

- You are kindly requested to note our reference format (more than 10 authors are listed in some of your citations) and update the list of references accordingly:
<http://www.embopress.org/page/journal/14693178/authorguide#referencesformat>.

- We noticed that Fig. EV4D is called out before EV4B; please make sure that Figure panels are called out in alphabetical order in your revised manuscript.

- Callouts for the Appendix Fig. S2A&B are missing. Please make sure that all figure panels are called out in your revised manuscript.

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Ioannis Papaioannou, PhD
Editor
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Referee #1:

The authors have done good revisions to address the issues that were raised by all reviewers.
There are many good aspects about this work but this reviewer believes that the authors should be cautious with the interpretation of their data. Not all the data are completely convincing and therefore several lines of interpretation are possible. The authors should make room for this. Actually, it is in the best interests of the authors not to overinterpret the data.

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The authors have addressed my concerns adequately through revisions and I recommend the manuscript should now be accepted for publication in EMBO Reports.

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As previously evaluated, most experiments are well designed and convincing, and this work will provide an important contribution in the field.

I have no major concern about the manuscript, even if I think that it will important in future investigations to determine precisely which activator(s) accumulates and is critical to control G2 phase duration in 2-cell embryos, according to the proposed model by the authors.
Also, it remains puzzling why CHK1 remains active in G2 phase during the second embryonic division.

Minor:
Lane 450: the sentence "when major genome activation is inhibited in Chk1 mKO, cleavage arrest depends on CHK1..." should be reformulated

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Referee #4:

The authors have done a lot of necessary additional experiments to confirm their findings. This effort substantially increased the quality of the ms. At the same time, the authors addressed all points/concerns I raised and therefore I recommend publication of the revised version.

Dear Dr. Papaioannou and Reviewers,

Enclosed, please find the revision of our manuscript entitled “CHK1-CDC25A-CDK1 regulate cell cycle progression and protect genome integrity in early mouse embryos”, submitted for consideration for publication in EMBO Reports. We thank the Reviewers for their valuable work, which helped us to improve the manuscript. We made our best effort to address the concerns raised in the review process; these changes are highlighted in green in the revised manuscript. What follows is a point-by-point response to the Reviewers’ concerns.

Sincerely yours,

David Drutovic

Referee #1:

The authors have done good revisions to address the issues that were raised by all reviewers. There are many good aspects about this work but this reviewer believes that the authors should be cautious with the interpretation of their data. Not all the data are completely convincing and therefore several lines of interpretation are possible. The authors should make room for this. Actually, it is in the best interests of the authors not to overinterpret the data.

We are pleased that the Reviewer found our story interesting. In order to avoid overinterpretation of our data, we have carefully revised the manuscript and modified some conclusions.

Referee #3:

As previously evaluated, most experiments are well designed and convincing, and this work will provide an important contribution in the field.

I have no major concern about the manuscript, even if I think that it will important in future investigations to determine precisely which activator(s) accumulates and is critical to control G2 phase duration in 2-cell embryos, according to the proposed model by the authors. Also, it remains puzzling why CHK1 remains active in G2 phase during the second embryonic division.

Minor:
Lane 450: the sentence "when major genome activation is inhibited in Chk1 mKO, cleavage arrest depends on CHK1..." should be reformulated

We modified the sentence. The text now reads:
Thus, when major genome activation is inhibited in *Chk1* mKO embryos by α-amanitin, maintaining of 2-cell stage block depends on CHK1 activity, but genome fragmentation is not dependent upon α-amanitin treatment.

Dr. David Drutovic
Institute of Animal Physiology and Genetics of the Czech Academy of Sciences
Rumburska 89
Libechov 27721
Czech Republic

Dear Dr. Drutovic,

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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The data shown in figures should satisfy the following conditions:

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

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

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- 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.
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- definitions of statistical methods and measures:

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