PPP4C facilitates homologous recombination DNA repair by dephosphorylating PLK1 during early embryo development

Ming-Zhe Dong, Ying-Chun Ouyang, Shi-Cai Gao, Xue-Shan Ma, Yi Hou, Heide Schatten, Zhen-Bo Wang and Qing-Yuan Sun
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MS TITLE: PPP4C facilitates homologous recombination DNA repair by dephosphorylating PLK1 during early embryo development

AUTHORS: Ming-Zhe Dong, Ying-Chun Ouyang, Shi-Cai Gao, Xue-Shan Ma, Yi Hou, Heide Schatten, Zhen-Bo Wang, and Qing-Yuan Sun

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing

how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The paper of Dong and colleagues investigates the role of PPP4C in connection with PLK1 and downstream signalling partners in DNA damage repair mechanism in early mouse embryos. While the role of PLK1 is well studied in context of cell cycle progression, and the phenotype of PPP4C deficient embryos has been shown before, it's role in homologous recombination (HR) step of DNA damage repair is less understood. The authors investigated the effects of the oocyte specific PPP4C knockout (ZP3-Cre induced) on HR in 2-cell stage embryos dissecting component of the signalling pathway involved in HR. These findings are novel and shed light on complicated mechanism of DNA damage repair and are valuable to the field.

Comments for the author

Introduction:
the first paragraph doesn't bring any essential information and could be easily skipped. Introduction could easily start from line 49.
Results (comments are organized by paragraphs)
Generation of Ppp4c conditional knockout mouse line The authors say that PPP4C expression is slightly decreased and located near the membrane 1 cell stage embryos. While this statement corresponds to the images shown in Fig S1B, I am not convinced this is a characteristic staining. Oocytes and zygotes tend to accumulate antibody particles in the cytoplasm in unspecific way. While this is not changing the outcome of any experiments, PPP4C expression should be evaluated more carefully. Was the dye swap performed and proper negative controls? If so, for the clarity it would be nice to see those in supplementary file, if not, the they should definitely be done. Fig1C shows MYC stain and the figure legend say it was performed after myc-ppp4c mRNA injection. I don’t see in the results section the explanation for this, it might be confusing for a reader not familiar with experimental approached adequate for early embryos. For Also labeling is a bit confusing, is should say myc-ppp4c instead of MYC.
PPP4C is crucial for female fertility but is not essential for oocyte meiotic maturation Line 142 should specify what kind of males were used.
Line 144 this sentence is out of place and belongs to materials and methods The results of the breeding assay at Fig 1D are presented in an unclear way to me. The way I see it (which is impossible) is that wild type mice gave 35 pups/female at 6 month of age. What is the number of pups per litter and how many litters were taken into account? I’d rather see a bar graph with this data. Another issue is the number of animals used in the study. N=4 per group is not enough to perform statistics with students t-test as indicated in materials and methods. Non parametric test should be used. In fact all statistics should be consulted with statistician.
Fig S2 uses Met II as an abbreviation of MII oocytes. This is rather strange and inconsistent with the text, therefore should be changed to MII.
Line 154 mentions “Ppp4c mutant”. For consistency Ppp4cfl/fl; Zp3-Cre or Ppp4cfl/fl should be used.
Loss of PPP4C leads to defective early embryonic development Line 154 “infertility” should be changed to sub-fertility.
On Fig2B the number of embryos should be shown (like it is on other graphs)
Depletion of PPP4C impairs genomic integrity in oocytes and fertilized eggs Fig3G a bright field picture with magnification equivalent to “merge” would be nice to see if possible.
Formation of micronuclei in PPP4C-deficient 2-cell embryos no comments
PPP4C deficiency induces sustaining CDK1 activity and influences RAD51 recruitment Line 216 “This demonstrated that DNA end resection had taken place and ssDNA invasion was influenced by PPP4C deficiency.” —> a more precise explanation is needed. “influenced” how?
Fig 5D-G WT and Zko/ZKO is used. For consistency Ppp4cfl/fl; Zp3-Cre or Ppp4cfl/fl should be used. Blots on G and H look rather strange. I would like to see the entire original picture Line 233 “influenced”—> how?

DNA damage in PPP4C-deficient embryos could be rescued by inhibition of PLK1 Fig6A —> this blot looks rather strange. I would like to see the entire original picture WT and Zko/ZKO is used. For consistency Ppp4cfl/fl; Zp3-Cre or Ppp4cfl/fl should be used.

DNA end resection requires PLK1 activity no comments
PPP4C interacts with PLK1 in the context of DNA damage, and knocking out PPP4C enhances PLK1 phosphorylation no comments
Phosphorylation of PLK1 at S137 and T210 influences RPA2 and RAD51 recruitment What are the normal zygotes in line 295. If Ppp4cfl/fl, this should be clearly stated.

Generally the words ”since” and “firstly” are miss-used through out the text.
Cells of 2 cell stage embryos are usually refereed as blastomeres and I would prefer that term rather than "cells".
General comment for figures:
-Figures contain scale bars of different length, thickness and style. Additionally, print on the sale bar in Fig4 and Fig5 is too small to read. All this should be unified and scale bar should be placed always at the same position on each picture on every figure (e.g. bottom right corner).
-Ppp4cfl/fl; Zp3-Cre or Ppp4cfl/fl on different graphs, different figures is either black or grey, this also should be the same on every graph/figure.
-Zko/ZKO is used or Ppp4cfl/fl; Zp3-Cre or Ppp4cfl/fl. All should be changed to Ppp4cfl/fl; Zp3-Cre or Ppp4cfl/fl for consistency (including supplementary files).
-I don’t see the reference for Fig9 in results or discussion section
Discussion:
Line 394, who/what is Adrian?
Some of the Ppp4cfl/fl; Zp3-Cre embryos reached the blastocyst stage, could authors speculate why that happened, where they phenotypically normal? Were the embryos recovered at 10.5 phenotypically normal? Was any stage after zygotic genome activation evaluated as well?
Materials ad Methods Should always mention the number of animals and oocytes/embryos used for each experiment.

Reviewer 2

Advance summary and potential significance to field

Dong and colleagues assess the role of the phosphatase, PPP4C, using a conditional knockout mouse approach and present that there is an accumulation of DNA damage in oocytes and early embryos. These defects are attributed to prolonged PLK1 activation, which led to prolonged CDK1 activity due to failure of CHK2 DNA damage response, increased BRCA2 phosphorylation, and inefficient loading of RAD51. The observations described are interesting and complement prior findings that used cell lines to describe the roles of PP4 in DNA damage response pathways. There are some critical aspects that need to be addressed.

Comments for the author

Major comments:
- As described, it is not clear how the breeding strategy was done. They need to explain this clearly instead of referring to “Mendel’s genetic law”.
- Supp Fig. 1A – The western blot needs to be quantified/normalized with the loading control. The pattern they describe may be due to less total protein present in the zygote lane.
- The specificity of their IF of PPP4C needs to be assessed using he Ppp4C conditional knockout (CKO) oocytes and embryos.
- The authors should assess PPP4C protein levels in the CKO, not just mRNA. They state they do this, but I don’t see it.
- The raw data for Fig. 1C needs to be given (i.e. data for each female).

Also, an indication of whether the successful births from the CKO resulted in developmental defects or were normal.
- The raw data for Fig. 2D needs to be given (i.e. data for each female).

Also, an indication of whether the successful births from the CKO resulted in developmental defects or were normal.

- The comet assay on GV and MII oocytes needs to be explained in more detail and extended with complementary assays. What is the significance of Fig.
3B not being different and Fig. 3C being different? Whereas there is difference in Fig. 3D for MII oocytes. The data suggests that increased DNA damage is happening somewhere during the GV to MII stage. However, it is not clear when.
For example, GVBD, MI, and MII oocytes should be assessed for gamma-H2AX. These could be done using whole cell IF or chromatin spread preparations (they have shown they can do chromatin spreads in Supp. Fig. S2D). Is the DNA damage occurring prior to meiosis I or is it following segregation of homologs? It is also confusing to me that there is unrepaired DNA damage at the GV stage considering the timing of the Zp3-Cre transgene. This requires more explanation.
- The authors need to quantify the PN2 stage oocytes as they have done for the PN5 in Fig. 3H. Especially considering "most of PP4C-deficient zygotes arrest at 2-cell stage". This would be a better path towards Fig. 4.
- The numbers in Fig. 3H are very low, only n=7 for control group.
- There is no explanation for the use of Etoposide in Fig. 4D and E or a comparison of findings for the NT and Etop group.
- The n values for a few important observations in Fig. 5B and C seem very low and should be expanded.
- A western blot of p-BRCA2 (S3291) is needed for the untreated CKO Vs control for Fig. 5F.
- The n values for a few very important observations in Fig. 6B and C are very low and should be expanded.
- Experiments in Fig. 6H and I need to be quantified.
- Figure 7A needs to be broken into 3x sub-figures (panels and 2x graphs).
Plus, it my be better to have these panels elsewhere, earlier in the paper.
- Figure 8B western blot needs to be quantified.
- Experiments in Fig. 8C and D need to be quantified.
- Couldn't the RPA accumulation represent incomplete DNA replication? The localization of RAD51 in that context is responding to this. The authors should assess whether markers of mitotic DNA synthesis are present during chromosome segregation (i.e. FANCD2).
- Antibodies should be given as a supplemental table with method(s) used for and dilution factors.
Minor comments:
- The delta symbol was not defined.
- Line 187 – Figure reference Fig. 4I is incorrect. Should be Fig. 3I.
- There are numerous times where spaces are missing in the text (e.g. line 199).

Reviewer 3

Advance summary and potential significance to field

This paper demonstrates that depletion of a protein phosphatase, termed PPP4C, in the oocyte leads to defects in DNA repair in the early embryo and implicates abnormal regulation of the phosphorylation state of polo-like kinase (PLK) 1 in this effect. This identifies Ppp4c as a maternal-effect gene and provides insight ito the regulation of DNA repair in the early mammalian embryo.

Comments for the author

Dong et al have studied the mechanisms the regulate DNA repair in early mammalian embryos, using embryos derived from eggs lacking a protein phosphatase, PPP4C, as an entry point. They conclude that PPP4C is required for efficient DNA repair, and does so thorough its activity to dephosphorylate PLK1.
This manuscript presents many interesting observations. In its present form however, it is almost impossible to follow. A number of factors contribute to this, including
i) the large number of proteins whose distribution or activity is studied (PPP4C, H2A.X, RPA2, RAD51, CHK1, CHK2, BRCA2, CDK1, CDC25B, PLK1). The relationship between these cannot easily be unravelled by the reader.

ii) using both the Ppp4c knockout and etoposide (and at one point also Xray), without clearly explaining the rationale for using the drug. Presumably, it is to increase DNA damage so the cellular response can be more easily monitored.
This is justifiable, but needs to be explained and the results need to be appropriately organized so the reader can follow the logic.
iii) whereas the first part of the paper focuses on CHK1, the second part focuses on CHK2. No explanation is given for the switch.
iv) a tendency to use words such as 'control' or 'regulate' rather than more specific terms such as 'activate' or 'inactivate' and 'phosphorylate' or
'dephosphorylate.' For example, the final sentence of the Abstract states that PPP4C is indispensable for the 'molecular dynamics' of PLK1. This unfortunately conveys no information. A specific statement is required.
To maximize the value of this contribution, the text needs to be extensively – probably almost completely – rewritten. Two suggestions that in my view will greatly improve it. First, the authors could construct a flow chart showing their view of how the 10 proteins listed above interact. Then the text could be written to show how each interaction in the chart is supported by the results. The cartoon in Figure 9 is not sufficiently specific – for example, neither CHK1 nor CHK2 are shown. Second, the authors should write from the perspective of understanding early embryonic development. The current version is more cell biological in orientation.
The authors are urged keep the following points in mind should they prepare a revised text.
l. 223 'In [knockout] embryos, CHK1 could be activated in response to DNA damage'. What evidence supports this assertion? Why do the authors rule out that the absence of PPP4C led to constitutive phosphorylation of CHK1 independent of DNA damage?
Fig. 7A shows H2A.X and RPA2 staining and quantification in wt and ko embryos.
This has previously been shown in Figs. 4 and 5. What is the reason to show it again?
l. 277. Increased binding after Xray is not evident in the image. The assertion that there is a difference should be supported by quantification of the signal and an appropriate number of biological replicates.
l. 277. Experiment used tissue culture cells, not 2-cell embryos.
l. 279. Define NICIF assay.
l. 284ff. State that PLK1 phosphorylation was increased in response to DNA damage in WT embryos, yet conclude that PPP4C dephosphorylates PLK1 in response to DNA damage. The observation and conclusion seem to be contradictory.

Fig. 3G. Has the transient increase in H2A.X at the 1-cell stage been previously described? If so, please cite. If not, please discuss why this might be occurring.
Fig. 3G. Increased H2A.X seems to be in male pronucleus. Was it seen in both male and female? This is important for interpreting the results, since it could be proposed that that PPP4C is needed to repair damaged DNA coming from the sperm. Alternatively, the DNA breaks could be related to DNA replication.
Along these lines, it would be interesting to compare the development of eggs (to blastocyst) of the two genotypes after parthenogenetic activation. If no difference, it would suggest that PPP4C is specifically needed to repair the damaged DNA provided by the sperm, adding substantially to the novelty and impact of this report.

The zeocin experiments (Fig. 3I, J) don't add much to the paper. Since oocyte maturation was monitored over a brief period of less than one day whereas embryo development over a longer period of 4-5 days, one cannot conclude that the zeomycin caused more severe damage to embryos than to oocytes.
Fig. 4D, E. The strategy behind the etoposide experiments has not previously been introduced or explained. Since there's no difference compared to control of the same genotype, what is the reason to show this data?
Why switch from CHK1 (up to Fig 5) to CHK2 (Fig 6 onwards)
L 244, Fig. 6D. Too strong to claim that CDK1 inhibitor rescued RAD51 recruitment. The magnitude of the difference between control and knockout is similar in the presence or absence of the RO336.
l. 121 Cannot conclude there is a decrease without more samples. Not important for the results, suggest simply omitting statement.
l. 125 Apparent peripheral accumulation of signal may be artifact caused by increasing specimen thickness towards middle. Again, not important fo the results, so better to delete.
l. 137 Knockout embryos are not shown in Fig S1A.

Fig. 3B, E What is the 'length rate.' Is it (tail length/total length)?
l. 187 Should be Fig. 3I
Fig 6B CDK inhibitor should be identified.

First revision

Author response to reviewers' comments

Responses to the reviewers' comments are in green.

Reviewer 1 Advance Summary and Potential Significance to Field:
The paper of Dong and colleagues investigates the role of PPP4C in connection with PLK1 and downstream signalling partners in DNA damage repair mechanism in early mouse embryos. While the role of PLK1 is well studied in context of cell cycle progression, and the phenotype of PPP4C deficient embryos has been shown before, it's role in homologous recombination (HR) step of DNA damage repair is less understood. The authors investigated the effects of the oocyte specific PPP4C knockout (ZP3- Cre induced) on HR in 2-cell stage embryos dissecting component of the signalling pathway involved in HR. These findings are novel and shed light on complicated mechanism of DNA damage repair and are valuable to the field.
Thank you very much for your positive comment.

Reviewer 1 Comments for the Author:
Introduction:
the first paragraph doesn't bring any essential information and could be easily skipped. Introduction could easily start from line 49.
We agree and have revised the manuscript according to your suggestion.

Results (comments are organized by paragraphs) Generation of Ppp4c conditional knockout mouse line
The authors say that PPP4C expression is slightly decreased and located near the membrane 1 cell stage embryos. While this statement corresponds to the images shown in Fig S1B, I am not convinced this is a characteristic staining. Oocytes and zygotes tend to accumulate antibody particles in the cytoplasm in unspecific way. While this is not changing the outcome of any experiments, PPP4C expression should be evaluated more carefully. Was the dye swap performed and proper negative controls? If so, for the clarity it would be nice to see those in supplementary file, if not, the they should definitely be done. Fig1C shows MYC stain and the figure legend say it was performed after myc-ppp4c mRNA injection. I don't see in the results section the explanation for this, it might be confusing for a reader not familiar with experimental approached adequate for early embryos. For Also labeling is a bit confusing, is should say myc-ppp4c instead of MYC.
We have revised our manuscript according to your suggestion. Please see lines 114-117 and Fig S1.

PPP4C is crucial for female fertility but is not essential for oocyte meiotic maturation
Line 142 should specify what kind of males were used.
We have specified the males according to your suggestion.

Line 144 this sentence is out of place and belongs to materials and methods.
We have revised the paper according to your suggestion.

The results of the breeding assay at Fig 1D are presented in an unclear way to me. The way I see it (which is impossible) is that wild type mice gave 35 pups/female at 6 month of age. What is the number of pups per litter and how many litters were taken into account? I'd rather see a bar graph with this data. Another issue is the number of animals used in the study. N=4 per group is not enough to perform statistics with students t-test as indicated in materials and methods. Non parametric test should be used. In fact all statistics should be consulted with statistician.
We have revised the paper according to your suggestion. Please see lines 134-137, Figure 1 and Table S1.

Fig S2 uses Met II as an abbreviation of MII oocytes. This is rather strange and inconsistent with the text, therefore should be changed to MII.
We have revised the manuscript according to your suggestion.

Line 154 mentions “Ppp4c mutant”. For consistency Ppp4cfl/fl; Zp3-Cre or Ppp4cfl/fl should be used.
We have revised the manuscript according to your suggestion.

Loss of PPP4C leads to defective early embryonic development Line 154 “infertility” should be changed to sub-fertility.
We apologize for the mistake; correction has been made.

On Fig2B the number of embryos should be shown (like it is on other graphs) We have revised the paper according to your suggestion.

Depletion of PPP4C impairs genomic integrity in oocytes and fertilized eggs Fig3G a bright field picture with magnification equivalent to “merge” would be nice to see if possible.
We have added a bright field picture of PN2 to PN5 in Figure S3B as suggested.

Formation of micronuclei in PPP4C-deficient 2-cell embryos no comments

PPP4C deficiency induces sustaining CDK1 activity and influences RAD51 recruitment
Line 216 “This demonstrated that DNA end resection had taken place and ssDNA invasion was influenced by PPP4C deficiency.” —> a more precise explanation is needed. “influenced” how?
We have revised the paper according to your suggestion.

Fig 5D-G WT and Zko/ZKO is used. For consistency Ppp4cfl/fl; Zp3-Cre or Ppp4cfl/fl should be used. Blots on G and H look rather strange. I would like to see the entire original picture
We have revised the paper according to your suggestion and added the entire original pictures in Figure S6

Line 233 “influenced”—> how?
We have added explanations according to your suggestion.

DNA damage in PPP4C-deficient embryos could be rescued by inhibition of PLK1
Fig6A —> this blot looks rather strange. I would like to see the entire original picture
We have added all of the original pictures in Figure S6

WT and Zko/ZKO is used. For consistency Ppp4cfl/fl; Zp3-Cre or Ppp4cfl/fl should be used.
It has been corrected.

DNA end resection requires PLK1 activity
no comments

PPP4C interacts with PLK1 in the context of DNA damage, and knocking out PPP4C enhances PLK1 phosphorylation no comments

Phosphorylation of PLK1 at S137 and T210 influences RPA2 and RAD51 recruitment
What are the normal zygotes in line 295. If Ppp4cfl/fl, this should be clearly stated.
It has been corrected.

Generally the words ”since” and “firstly” are miss-used through out the text.
We have asked Prof. Heide Schatten, one of the co-authors, to pay careful attention to the wording.

Cells of 2 cell stage embryos are usually refereed as blastomeres and I would prefer that term rather than "cells".
We think “blastomere” is a broad definition, including not only 2-cell, but also 4-cell, 8-cell stages. So “2-cell stage or 2-cell embryo” may be more accurate.

General comment for figures:
-Figures contain scale bars of different length, thickness and style. Additionally, print on the sale bar in Fig4 and Fig5 is too small to read. All this should be unified and scale bar should be placed always at the same position on each picture on every figure (e.g. bottom right corner).
It has been corrected according to your suggestion.

-Ppp4cfl/fl; Zp3-Cre or Ppp4cfl/fl on different graphs, different figures is either black or grey, this also should be the same on every graph/figure.
It has been corrected according to your suggestion.

-Zko/ZKO is used or Ppp4cfl/fl; Zp3-Cre or Ppp4cfl/fl. All should be changed to Ppp4cfl/fl; Zp3-Cre or Ppp4cfl/fl for consistency (including supplementary files).
It has been corrected according to your suggestion.

-I don't see the reference for Fig9 in results or discussion section
We have re-written this part according to your suggestion.

Discussion:
Line 394, who/what is Adrian?
We made the appropriate revisions.

Some of the Ppp4cfl/fl; Zp3-Cre embryos reached the blastocyst stage, could authors speculate why that happened, where they phenotypically normal?
Were the embryos recovered at 10.5 phenotypically normal? Was any stage after zygotic genome activation evaluated as well?
We have revised the paper according to your suggestion and added the explanation in lines 393-396.

Materials ad Methods
Should always mention the number of animals and oocytes/embryos used for each experiment.
We have revised the paper according to your suggestion. Special thanks to you for your good comments.

Reviewer 2 Advance Summary and Potential Significance to Field:
Dong and colleagues assess the role of the phosphatase, PPP4C, using a conditional knockout mouse approach and present that there is an accumulation of DNA damage in oocytes and early embryos. These defects are attributed to prolonged PLK1 activation, which led to prolonged CDK1 activity due to failure of CHK2 DNA damage response, increased BRCA2 phosphorylation, and inefficient loading of RAD51. The observations described are interesting and complement prior findings that used cell lines to describe the roles of PP4 in DNA damage response pathways. There are some critical aspects that need to be addressed.
Thank you very much for your positive comment.

Reviewer 2 Comments for the Author:
Major comments:
•As described, it is not clear how the breeding strategy was done. They need to explain this clearly instead of referring to "Mendel's genetic law".
We have added the explanation according to your suggestion. Please see lines 123-128.

•Supp Fig. 1A – The western blot needs to be quantified/normalized with the loading control. The pattern they describe may be due to less total protein present in the zygote lane.
We have revised the paper according to your suggestion.

•The specificity of their IF of PPP4C needs to be assessed using he Ppp4C conditional knockout (CKO) oocytes and embryos.
We have repeated the experiment about subcellular localization of PPP4C. PPP4C was distributed in both nuclei and cytoplasm during oocyte meiotic maturation and embryo development without specific localization. The previous result describing PPP4C location near the membrane of 1 cell stage embryos might be due to the slide preparation technique.

•The authors should assess PPP4C protein levels in the CKO, not just mRNA. They state they do this, but I don't see it.
We addressed this question. Please see Figure 1C

•The raw data for Fig. 1C needs to be given (i.e. data for each female). Also, an indication of whether the successful births from the CKO resulted in developmental defects or were normal.
We believe your mention of Fig. 1C may be Fig. 1D. The raw data is provided in Table S1.

•The raw data for Fig. 2D needs to be given (i.e. data for each female). Also, an indication of whether the successful births from the CKO resulted in developmental defects or were normal.
The raw data was provided in Table S2. We did not find developmental defects in the successful births from the CKO; unfortunately, we did not collect this data.

•The comet assay on GV and MII oocytes needs to be explained in more detail and extended with complementary assays. What is the significance of Fig.3B not being different and Fig. 3C being different? Whereas there is difference in Fig. 3D for MII oocytes. The data suggests that increased DNA damage is happening somewhere during the GV to MII stage. However, it is not clear when. For example, GVBD, MI, and MII oocytes should be assessed for gamma-H2AX. These could be done using whole cell IF or chromatin spread preparations (they have shown they can do chromatin spreads in Supp. Fig. S2D). Is the DNA damage occurring prior to meiosis I or is it following segregation of homologs? It is also confusing to me that there is unrepaired DNA damage at the GV stage considering the timing of the Zp3- Cre transgene. This requires more explanation.
Fig. 3B means the tail length/total length, and Fig, 3C means the proportion of comet tail DNA. As shown in Fig. 3A, some GV oocytes of WT had elongated comet tails, but the GV oocytes of the Zko group had wide and long comet tails. So, the proportion of comet tail DNA in Zko is increased. We have carried out the experiment according to your suggestion, Unfortunately, as shown in Fig. S3A, γH2AX was not a good marker that indicated DNA damage in metaphase.

•The authors need to quantify the PN2 stage oocytes as they have done for the PN5 in Fig. 3H. Especially considering "most of PP4C-deficient zygotes arrest at 2-cell stage". This would be a better path towards Fig. 4.
We have added quantifying data of the PN2 stage zygote and the data are shown in Fig. 3H.

•The numbers in Fig. 3H are very low, only n=7 for control group. We have repeated the experiment and added the numbers.

•There is no explanation for the use of Etoposide in Fig. 4D and E or a comparison of findings for the NT and Etop group.
We have deleted the Etop group according to your suggestion.

•The n values for a few important observations in Fig. 5B and C seem very low and should be expanded.
We have repeated the experiment and expanded the numbers.

•A western blot of p-BRCA2 (S3291) is needed for the untreated CKO Vs control for Fig. 5F.
We have added this data in Fig. S4B.

•The n values for a few very important observations in Fig. 6B and C are very low and should be expanded.
We have repeated the experiment and expanded the numbers as suggested.

•Experiments in Fig. 6H and I need to be quantified.
These experiments have been quantified and the data are shown in Fig. 6.

•Figure 7A needs to be broken into 3x sub-figures (panels and 2x graphs). Plus, it my be better to have these panels elsewhere, earlier in the paper.
It has been corrected according to your suggestion.

•Figure 8B western blot needs to be quantified.
These experiments have been quantified and the data are shown in Fig. 8.

•Experiments in Fig. 8C and D need to be quantified.
These experiments have been quantified and the data has been shown in Fig. 8.

•Couldn't the RPA accumulation represent incomplete DNA replication? The localization of RAD51 in that context is responding to this. The authors should assess whether markers of mitotic DNA synthesis are present during chromosome segregation (i.e. FANCD2).
Corresponding experiments have been carried out according to your suggestion and data are shown in Fig. S4A.

•Antibodies should be given as a supplemental table with method(s) used for and dilution factors.
We have revised the paper according to your suggestion. Antibodies and reagents are shown in Table. S3.

Minor comments:
•The delta symbol was not defined.
It has been corrected as suggested. Line128.

•Line 187 – Figure reference Fig. 4I is incorrect. Should be Fig. 3I. It has been corrected as suggested.

•There are numerous times where spaces are missing in the text (e.g. line 199).
It has been corrected as suggested.

Special thanks to you for your good comments.

Reviewer 3 Advance Summary and Potential Significance to Field:
This paper demonstrates that depletion of a protein phosphatase, termed PPP4C, in the oocyte leads to defects in DNA repair in the early embryo and implicates abnormal regulation of the phosphorylation state of polo-like kinase (PLK) 1 in this effect. This identifies Ppp4c as a maternal-effect gene and provides insight ito the regulation of DNA repair in the early mammalian embryo.
Thank you very much for your positive comment.

Reviewer 3 Comments for the Author:
Dong et al have studied the mechanisms the regulate DNA repair in early mammalian embryos, using embryos derived from eggs lacking a protein phosphatase, PPP4C, as an entry point. They conclude that PPP4C is required for efficient DNA repair, and does so thorough its activity to dephosphorylate PLK1. This manuscript presents many interesting observations. In its present form, however, it is almost impossible to follow. A number of factors contribute to this, including

i) the large number of proteins whose distribution or activity is studied (PPP4C, H2A.X, RPA2, RAD51, CHK1, CHK2, BRCA2, CDK1, CDC25B, PLK1). The relationship between these cannot easily be unravelled by the reader.
We have re-written the discussion (line 260-279) and revised Fig. 9 to help the reader to better understand the relationship between these proteins according to your suggestion.

ii) using both the Ppp4c knockout and etoposide (and at one point also Xray), without clearly explaining the rationale for using the drug. Presumably, it is to increase DNA damage so the cellular response can be more easily monitored. This is justifiable, but needs to be explained and the results need to be appropriately organized so the reader can follow the logic.
We have added an explanation that this treatment is to induce lesions. Please see lines 206 and 232.

iii) whereas the first part of the paper focuses on CHK1, the second part focuses on CHK2. No explanation is given for the switch.
We have added the explanation about the relationship in the discussion, hoping that it will be helpful to the reader.

iv) a tendency to use words such as ‘control’ or ‘regulate’ rather than more specific terms such as ‘activate’ or ‘inactivate’ and ‘phosphorylate’ or ‘dephosphorylate.’ For example, the final sentence of the Abstract states that PPP4C is indispensable for the ‘molecular dynamics’ of PLK1. This unfortunately conveys no information. A specific statement is required.
We have revised that paper according to your suggestion.

To maximize the value of this contribution, the text needs to be extensively – probably almost completely – rewritten. Two suggestions that in my view will greatly improve it. First, the authors could construct a flow chart showing their view of how the 10 proteins listed above interact. Then the text could be written to show how each interaction in the chart is supported by the results.
The cartoon in Figure 9 is not sufficiently specific – for example, neither CHK1 nor CHK2 are shown. Second, the authors should write from the perspective of understanding early embryonic development. The current version is more cell biological in orientation. The authors are urged keep the following points in mind should they prepare a revised text.

Thank you very much for your suggestion. As suggested, we have re-written some parts and included the discussion from the perspective of understanding early embryonic development in the revised version. We have asked Prof.
Heide Schatten to edit the revised manuscript. Figure 9 is improved as well.

l. 223 ‘In [knockout] embryos, CHK1 could be activated in response to DNA damage’. What evidence supports this assertion? Why do the authors rule out that the absence of PPP4C led to constitutive phosphorylation of CHK1 independent of DNA damage?
Because we have observed that DNA damage had existed in PPP4C deficient oocytes and embryos as shown in Fig. 3 and Fig. 4B-D. So CHK1 was activated in Zko embryos (Fig. 5D)and is dependent on DNA damage. We thought this is normal.

Fig. 7A shows H2A.X and RPA2 staining and quantification in wt and ko embryos. This has previously been shown in Figs. 4 and 5. What is the reason to show it again? Figure 7A G1, Figs 4 and 5 G2.
Figs. 4 and 5 show H2A.X and RPA2 staining and quantification in G2 phase, while Fig. 7A shows these in the G1 phase to prove that DNA end resection requires PLK1 activity.

l. 277. Increased binding after Xray is not evident in the image. The assertion that there is a difference should be supported by quantification of the signal and an appropriate number of biological replicates.
We have deleted this sentence.

l. 277. Experiment used tissue culture cells, not 2-cell embryos.
Generally, IP need huge amounts of protein, however, 2-cell embryos cannot satisfy this purpose.

l. 279. Define NICIF assay.
We have added the definition according to your suggestion in lines 276-277.

l. 284ff. State that PLK1 phosphorylation was increased in response to DNA damage in WT embryos, yet conclude that PPP4C dephosphorylates PLK1 in response to DNA damage. The observation and conclusion seem to be contradictory.
We have added an explanation according to your suggestion in lines 282-291.

Fig. 3G. Has the transient increase in H2A.X at the 1-cell stage been previously described? If so, please cite. If not, please discuss why this might be occurring.
We have cited Xu et al., 2015. Please see line 175.

Fig. 3G. Increased H2A.X seems to be in male pronucleus. Was it seen in both male and female? This is important for interpreting the results, since it could be proposed that that PPP4C is needed to repair damaged DNA coming from the sperm. Alternatively, the DNA breaks could be related to DNA replication. Along these lines, it would be interesting to compare the development of eggs (to blastocyst) of the two genotypes after parthenogenetic activation. If no difference, it would suggest that PPP4C is specifically needed to repair the damaged DNA provided by the sperm,

adding substantially to the novelty and impact of this report.
As shown in Figs. 3G and S3B, we think increasedγH2AX is random between male and female pronucleus.

The zeocin experiments (Fig. 3I, J) don't add much to the paper. Since oocyte maturation was monitored over a brief period of less than one day whereas embryo development over a longer period of 4-5 days, one cannot conclude that the zeomycin caused more severe damage to embryos than to oocytes.
We have deleted this data taking your comment into consideration.

Fig. 4D, E. The strategy behind the etoposide experiments has not previously been introduced or explained. Since there's no difference compared to control of the same genotype, what is the reason to show this data?
We have deleted this data taking you're your comment into consideration.

Why switch from CHK1 (up to Fig 5) to CHK2 (Fig 6 onwards)
Because both CHK1 and CHK2 are the upstream regulators of CDK1. In PPP4C-deficient 2-cell embryos, CHK1 could be duly activated in response to DNA damage (Fig. 5D, E). So we speculated that CHK2 may not be activated in response to DNA damage.

L 244, Fig. 6D. Too strong to claim that CDK1 inhibitor rescued RAD51 recruitment. The magnitude of the difference between control and knockout is similar in the presence or absence of the RO336.
We have repeated this experiment and expanded the numbers.

l. 121 Cannot conclude there is a decrease without more samples. Not important for the results, suggest simply omitting statement.
We have quantified and revised the conclusion.

l. 125 Apparent peripheral accumulation of signal may be artifact caused by increasing specimen thickness towards middle. Again, not important fo the results, so better to delete.
We have repeated this experiment and made revisions.

l. 137 Knockout embryos are not shown in Fig S1A.
We apologize for the incorrect writing and have included it in the revised manuscript.

Fig. 3B, E What is the 'length rate.' Is it (tail length/total length)? We have added it in the revised manuscript.

l. 187 Should be Fig. 3I
We apologize for our incorrect writing and have made corrections.

Fig 6B CDK inhibitor should be identified.
We have revised the Fig. 6B legend according to your suggestion. Special thanks to you for your good comments.

We used extended time to do some additional experiments or repeat some experiments, and tried our best to improve the manuscript. The changes were marked in red in the revised paper. We appreciate the Reviewers' hard work and helpful suggestions, and we hope that our corrections will have improved the manuscript.

Second decision letter

MS ID#: DEVELOP/2021/200351

MS TITLE: PPP4C facilitates homologous recombination DNA repair by dephosphorylating PLK1 during early embryo development

AUTHORS: Ming-Zhe Dong, Ying-Chun Ouyang, Shi-Cai Gao, Xue-Shan Ma, Yi Hou, Heide Schatten, Zhen-Bo Wang, and Qing-Yuan Sun

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

Reviewer 1

Advance summary and potential significance to field

The authors have revised the manuscript and sufficiently addressed my comments.

Comments for the author

I do have two remarks still.
In table S1: "letters/mother" I believe it should be "litters/mother".
Line 392: ZP3Cre is known to be leaky, therefore it is more likely that the E10.5 embryos are side effect of ZP3Cre leakiness.

Reviewer 2

Advance summary and potential significance to field

Thank you for covering the suggested edits. I think the finding that DNA replication is incomplete is a very important finding for future work.

Comments for the author

You may wish to incorporate two recent publications on PLK1 and role in DNA damage response in mitotic and meiotic cells. This will bolster your discussion:
https://pubmed.ncbi.nlm.nih.gov/35119917/
https://www.molbiolcell.org/doi/abs/10.1091/mbc.E21-03-0115

Reviewer 3

Advance summary and potential significance to field

This paper demonstrates that depletion of a protein phosphatase, termed PPP4C, in the oocyte leads to defects in DNA repair in the early embryo and implicates abnormal regulation of the phosphorylation state of polo-like kinase (PLK) 1 in this effect. This identifies Ppp4c as a maternal-effect gene and provides insight ito the regulation of DNA repair in the early mammalian embryo.

Comments for the author

This revised version is much improved, though remains dense and difficult to follow. Points that should be addressed include:
l. 181. “As most of PPP4C-deficient zygotes were arrested at the 2-cell stage…”
Not supported by Fig. 2B, which shows considerably less than half arrested at the 2-cell stage. Minor note, zygote refers only to the 1-cell stage; beyond this point, they should be termed embryos.
l. 184. BrdU staining procedure not described in the Methods.
l. 244. “Inhibition of CDK1 could not eliminate…” Not supported by the data in Fig. 6D.
l. 272. Cell type and methods used for the IP needs to be described. Nothing in the text indicates that tissue-culture cells rather than embryos were used here.
l. 275. “We observed clear immunoreactivity…” This description of co-ip needs to be revised.
l. 283. “…decreased when treated with Etoposide…” Statement seems to be based on a single experiment where a mild difference was seen. Multiple replicates with mean and SEM required followed by statistical test.
l. 293 ff. An explanation of the mutants and their effects on PLK activity needs to be provided.
Fig. 8C. Images are of poor quality.
General point Methods are rather superficially described in many places. As one example, were the mutant forms pf PLK1 sequences to verify that the expected base-changes had occurred? How was the concentration of the transcribed mRNA measured?

Second revision

Author response to reviewers' comments

Responses to the reviewers’ comments are in green.

Reviewer 1 Advance Summary and Potential Significance to Field:
The authors have revised the manuscript and sufficiently addressed my comments.
Thank you very much for your positive comment.

Reviewer 1 Comments for the Author:
I do have two remarks still.
In table S1: "letters/mother" I believe it should be "litters/mother".
We apologize for our incorrect writing and have made corrections.

Line 392: ZP3Cre is known to be leaky, therefore it is more likely that the E10.5 embryos are side effect of ZP3Cre leakiness.
We have added this viewpoint in discussion according to your suggestion. Please see lines 399-401.

Reviewer 2 Advance Summary and Potential Significance to Field:
Thank you for covering the suggested edits. I think the finding that DNA replication is incomplete is a very important finding for future work.
Thank you very much for your positive comment.

Reviewer 2 Comments for the Author:
You may wish to incorporate two recent publications on PLK1 and role in DNA damage response in mitotic and meiotic cells. This will bolster your discussion:

https://pubmed.ncbi.nlm.nih.gov/35119917/

https://www.molbiolcell.org/doi/abs/10.1091/mbc.E21-03-0115
We have incorporated these two recent publications in our discussion according to your suggestion. Please see lines 337-340.

Reviewer 3 Advance Summary and Potential Significance to Field:
This paper demonstrates that depletion of a protein phosphatase, termed PPP4C, in the oocyte leads to defects in DNA repair in the early embryo and implicates abnormal regulation of the phosphorylation state of polo-like kinase (PLK) 1 in this effect. This identifies Ppp4c as a maternal-effect gene and provides insight to the regulation of DNA repair in the early mammalian embryo.
Thank you very much for your positive comment.

Reviewer 3 Comments for the Author:
This revised version is much improved, though remains dense and difficult to follow. Points that should be addressed include:
l. 181. "As most of PPP4C-deficient zygotes were arrested at the 2-cell stage…"
Not supported by Fig. 2B, which shows considerably less than half arrested at the 2-cell stage. Minor note, zygote refers only to the 1-cell stage; beyond
this point, they should be termed embryos.
We have revised this section.

l. 184. BrdU staining procedure not described in the Methods.
We have added BrdU staining procedure in the Methods according to your suggestion. Please see line 459-467.

l. 244. "Inhibition of CDK1 could not eliminate…" Not supported by the data in Fig. 6D.
We have deleted this sentence.

l. 272. Cell type and methods used for the IP needs to be described. Nothing in the text indicates that tissue-culture cells rather than embryos were used here.
We have revised this sentence and added cell type and methods. Please see line 269-270.

l. 275. "We observed clear immunoreactivity…" This description of co-ip needs to be revised.
We have revised this description according to your suggestion.

l. 283. "…decreased when treated with Etoposide…" Statement seems to be based on a single experiment where a mild difference was seen. Multiple replicates with mean and SEM required followed by statistical test.
We have repeated this experiment twice again and made revisions.

l. 293 ff. An explanation of the mutants and their effects on PLK activity needs to be provided.
Fig. 8C. Images are of poor quality.
We have added an explanation according to your suggestion in lines 290-294. The images in Fig. 8C have been changed.

General point
Methods are rather superficially described in many places. As one example, were the mutant forms pf PLK1 sequences to verify that the expected base- changes had occurred? How was the concentration of the transcribed mRNA measured?
The methods have been supplemented according to your suggestion, the primers were provided in Table S4.

Third decision letter

MS ID#: DEVELOP/2021/200351

MS TITLE: PPP4C facilitates homologous recombination DNA repair by dephosphorylating PLK1 during early embryo development

AUTHORS: Ming-Zhe Dong, Ying-Chun Ouyang, Shi-Cai Gao, Xue-Shan Ma, Yi Hou, Heide Schatten, Zhen-Bo Wang, and Qing-Yuan Sun
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.