DPPA2 and DPPA4 are dispensable for mouse zygotic genome activation and pre-implantation development

Zhiyuan Chen, Zhenfei Xie and Yi Zhang
DOI: 10.1242/dev.200178

Editor: Patrick Tam

Review timeline
Original submission: 8 September 2021
Editorial decision: 27 September 2021
First revision received: 9 November 2021
Accepted: 25 November 2021

Original submission

First decision letter

MS ID#: DEVELOP/2021/200178

MS TITLE: DPPA2 and DPPA4 are dispensable for mouse zygotic genome activation and preimplantation development

AUTHORS: Zhiyuan Chen, Zhenfei Xie, and Yi Zhang

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 interest in your work, but have requested clarification of the role of Dppa2 and -4 on post-blastocyst development of double knockout embryos (reviewers 1 and 3, see Editor's note), and provision of missing information on methodology and in the dataset. On these considerations, the reviewers recommend a substantial revision of your manuscript before it can be considered for 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 major wave of zygotic genome activation (ZGA) begins at the 2-cell stage and is essential for early embryo development. 2C-like ES cells represent a small subpopulation of mouse embryonic stem (ES) cells and share some genetic and epigenetic features with mouse 2-cell embryos. Results from 2C-like ES cells have been used as proxies for 2-cell embryos. Developmental Pluripotency Associated 2 (Dppa2) and 4 (Dppa4) were previously identified activators of Dux expression and potent inducers of the 2C-like state. Their ablation affects differentiation of ES cells in vitro.

In this manuscript, the authors document that Dppa2 and Dapp4 are not required for pre-implantation development in vivo using gene-edited mice.
The authors established individual Dppa2 and Dppa4 maternal (or maternal and zygotic) KO lines and documented that all embryos developed into blastocysts after in vitro fertilization (IVF).
Applying single embryo RNA-seq to WT and KO 2-cell embryos, the authors determined that maternal and zygotic deletion of Dppa2 or Dppa4 did not affect Dux expression, nor many other ZGA genes.
Comparing their RNA-seq data to previously published results in 2C-like ES cells with Dppa2/4 depletion, the authors determined that 2C-like ES cells did not recapitulate 2-cell embryos in ZGA gene regulation and raised concerns of the validity of extrapolating results from 2C-like ES cells to early embryos.

Comments for the author

The experiments were carefully designed and interpreted. Although immunostaining indicated that depletion of one gene affects expression of the other, the manuscript lacks Dppa2 and Dppa4 double knockout mice. The knockout embryos in the study were generated by IVF and embryos flushed from the reproductive tract after natural mating should be analyzed to confirm in vivo functions of Dppa2 and Dppa4.
Other issues that the authors may wish to address include:
1. Fig. 1C, 2D and 3A: Data from more embryos would provide more robust results.
2. In Fig. 3A, the genotypes of single embryos in the m-z+/m-z- groups should be provided to confirm the expected Mendelian ratio of embryos at later stages (e.g., morula and blastocyst). Only if correct Mendelian ratios pertain can the authors conclude that Dppa2 or Dppa4 ablation does not affect pre-implantation development.
3. The experiments in Fig. 3A should be repeated using natural mated females.
4. Resolution of photos in Fig. 3B is poor: can better images be provided?
5. The litter size and genotypes of pups should be provided.
6. In Fig. 4A, do all genomic views should use the same scale on the y axis?
If a different scale is used, please clarify in figure or legend.
7. Clarify what is meant by ‘repeat’ in the Fig. 4B legend and text. Why are there fewer datapoints in the repeat plots? Also, why not show all the genes listed in Fig. 4B in all 4 panels?
8. Same question (#7) for Fig. S3C.
9. In Fig. 4D, why not provide heatmap of more genes since the whole transcriptomes are sequenced? Results from more genes would make the conclusion more convincing.
10. MuERV-L-Gag is widely used a marker for ZGA. Immunostaining of MuERV-L-Gag and/or other ZGA markers in WT embryo or embryos with Dppa2/4 depletion would provide more direct evidence for Dppa2/4 functions in ZGA and should be included in Fig. 4.
11. Are ERCC spike-ins used for the RNA-seq experiments? If so, are they used for normalization of gene expression in different samples? Please provide more detail in “RNA-Seq libraries preparation and data processing”
of Methods

Reviewer 2

Advance summary and potential significance to field

In this manuscript, Chen et al. explore the potential role of DPPA2 and DPPA4 during preimplantation mouse development. They first summarise RNA expression from the literature and characterise protein distribution of both factors using immunohistochemistry in early embryos. Gdf9-Cre was employed to enable recombination of LoxP sites in either gene during early oocyte development: Dppa4 targeting was obtained courtesy of the authors of a previously published study but Dppa2 fl/fl was generated and validated de novo by the present authors, adding a novel mouse line for the community. The mating scheme used to produce the compound deletion and control embryos is clearly presented. Quite surprisingly and of interest to the field, the authors found that there was little effect of mutation of either DPPA2 or 4 on preimplantation mouse development. They confirm using immunohistochemistry the previous assumption, based upon the known operation of DPPA2 and 4 as heterodimers, that loss of one protein causes reciprocal reduction of the other, and thereby conclude that neither DPPA2 nor DPPA4 is required for early mouse development. This is an important finding because previous published studies using embryonic stem cells as a proxy for the embryo made the opposite assumption. We can conclude that, particularly in the context of cleavage stages of mammalian development, ESCs are not a good model, even if they can be shown to self-organise into structures resembling blastocysts.

Comments for the author

1.The materials and methods section and figure legends are very brief and more information should be provided. For example, how were the embryos in Fig.3B cultured? Were those embryos used for the IHC in Fig.3C? If not, were they flushed as blastocysts or cultured from earlier stages? The reader is unlikely to find out this information from the papers quoted in the methods, so a more detailed description is needed.
2.In Fig.3B, could the authors explain why the m-z+ and m-z- embryos pooled?
Note to the authors for future work:
The use of tail tips for genotyping is generally discouraged these days, since the optimisation of PCR genotyping techniques means that a small ear biopsy provides ample material and is less invasive to the animal.

Reviewer 3

Advance summary and potential significance to field

Which maternal factors initiate zygotic genome activation (ZGA) remains a critical question for preimplantation development. Recently, 2C-like cells have emerged as a novel model to study ZGA. A novel TF that mediates ZGA, Dux, is identified through this model. However, Dux is not a maternal factor, and the maternal factors that activate Dux remain identified.
Through the 2C-like cell model, Dppa2/4 has been identified as the transcription factor that activates Dux in mESCs. In addition, several studies provide evidence suggesting that Dppa2/4 plays a role in preimplantation development.
All these indicate that Dppa2/4 is a maternal factor contributing to ZGA.
Nevertheless, whether Dppa2/4 are crucial in ZGA has not been rigorously examined and remains an important question in the preimplantation development study.

Comments for the author

In this manuscript, Chen et al. provide the first evidence proving that Dppa2/4 are dispensable for ZGA. Their results suggest that Dppa2/4 is barely present in embryos during ZGA. The absence of Dppa2/4 shows minimum effects on the activation of ZGA genes, including Dux and transcripts activated by Dux. These results are unexpected but provide a piece of crucial information to the preimplantation development field.
The data presented in this manuscript are concrete, well-organized, and sufficient to support their statements. I only have some minor comments.
1. Dppa2/4 shapes the DNA methylome of mESCs (Eckersley-Maslin, 2020; Eckersley-

Maslin et al., 2020; Gretarsson and Hackett, 2020). Although Dppa2/4 is not present after implantation, the KO of Dppa2/4 impairs the post-implantation development, indicating that Dppa2/4 KO causes epigenetic alterations in blastocysts. Therefore, it would be nice should the authors provide the DNA methylome alterations in Dppa2/4 KO blastocysts and speculate how these alterations impairs embryonic development.
2. Several previous studies (Hu et al. 2010; Yan et al. 2019) suggest that Dppa2/4 are functionally important for ZGA. Thus, I recommend that the authors provide a detailed discussion on the inconsistency between their results and previous studies.

First revision

Author response to reviewers' comments

Responses to the Reviewers' comments

We thank the reviewers for their insightful comments. We address their comments point-by-point below.

Editor's note:

Please provide data on the genotypes of in vitro cultured embryos at the late blastocyst and mutant blastocysts of natural mating to confirm that Dppa2 and Dppa4 are not required for pre-implantation development.

Response: We have performed the suggested experiments and analyses. More detailed information can be found in our responses to Comment #2 and #4 of Reviewer #1.

Reviewer 1 Advance Summary and Potential Significance to Field:
The major wave of zygotic genome activation (ZGA) begins at the 2-cell stage and is essential for early embryo development. 2C-like ES cells represent a small subpopulation of mouse embryonic stem (ES) cells and share some genetic and epigenetic features with mouse 2-cell embryos. Results from 2C-like ES cells have been used as proxies for 2-cell embryos. Developmental Pluripotency Associated 2 (Dppa2) and 4 (Dppa4) were previously identified activators of Dux expression and potent inducers of the 2C-like state. Their ablation affects differentiation of ES cells in vitro.

In this manuscript, the authors document that Dppa2 and Dapp4 are not required for pre-implantation development in vivo using gene-edited mice. The authors established individual Dppa2 and Dppa4 maternal (or maternal and zygotic) KO lines and documented that all embryos developed into blastocysts after in vitro fertilization (IVF). Applying single embryo RNA-seq to WT and KO 2-cell embryos, the authors determined that maternal and zygotic deletion of Dppa2 or Dppa4 did not affect Dux expression, nor many other ZGA genes. Comparing their RNA-seq data to previously published results in 2C-like ES cells with Dppa2/4 depletion, the authors determined that 2C-like ES cells did not recapitulate 2-cell embryos in ZGA gene regulation and raised concerns of the validity of extrapolating results from 2C-like ES cells to early embryos.

We'd like to thank the reviewer for very nicely summarizing our work.

Reviewer 1 Comments for the Author:

Comment #1: The experiments were carefully designed and interpreted. Although immunostaining indicated that depletion of one gene affects expression of the other, the manuscript lacks Dppa2 and Dppa4 double knockout mice.

Response: We agree that double KO (DKO) would be the definite evidence to support that Dppa2 and Dppa4 are dispensable for mouse preimplantation development. However, generating DKO by natural mating using our mutants is infeasible because *Dppa2* and *Dppa4* are genetically closely linked. Importantly, we believe that adding Dppa2/4 DKO should not change our conclusion for

following three reasons. First, Dppa2 and Dppa4 are well known to function as a heterodimer and depletion of either one abolishes the heterodimer function (Hernandez et al., 2018). Second, depletion of Dppa4 results in almost complete depletion (>90%) of Dppa2 at protein level in both embryos and ESCs (Gretarsson and Hackett, 2020)(**Fig. 2C, 2D** and revised **Fig. 3B, 3D**). Third, zygotic KO of individual Dppa2 or Dppa4 have nearly identical phenotypic defects to zygotic DKO mice (Nakamura et al., 2011). Thus, we believe that our manuscript in current form should be complete and strongly supports the dispensable role of Dppa2/4 in ZGA and preimplantation embryos.

Comment #2: The knockout embryos in the study were generated by IVF and embryos flushed from the reproductive tract after natural mating should be analyzed to confirm in vivo functions of Dppa2 and Dppa4.

Response: We have analyzed *in vivo* blastocysts from natural mating as suggested. As shown in **Fig. R1** (next page), loss of maternal and zygotic Dppa2 or Dppa4 has no effect on preimplantation development. These data have been included in the revised manuscript (revised **Fig 3** and **Fig. S2A**, Line 159-161).

A

Dppa2/4 in vivo E3.5 blastocysts collected after natural mating

Mating pairs	Blastocysts/litters	Blastocysts ± SD	m-z- (%)
Dppa2fl/fl × Dppa2+/-	18/3	6.0 ± 1.7	0 (0)
Gdf9-Cre,Dppa2fl/fl × Dppa2+/-	21/3	7.0 ± 1.0	9 (42.8)
Dppa4fl/fl × Dppa4+/-	19/4	4.8 ± 0.9	0 (0)
Gdf9-Cre,Dppa4fl/fl × Dppa4+/-	17/3	5.6 ± 1.5	9 (52.9)

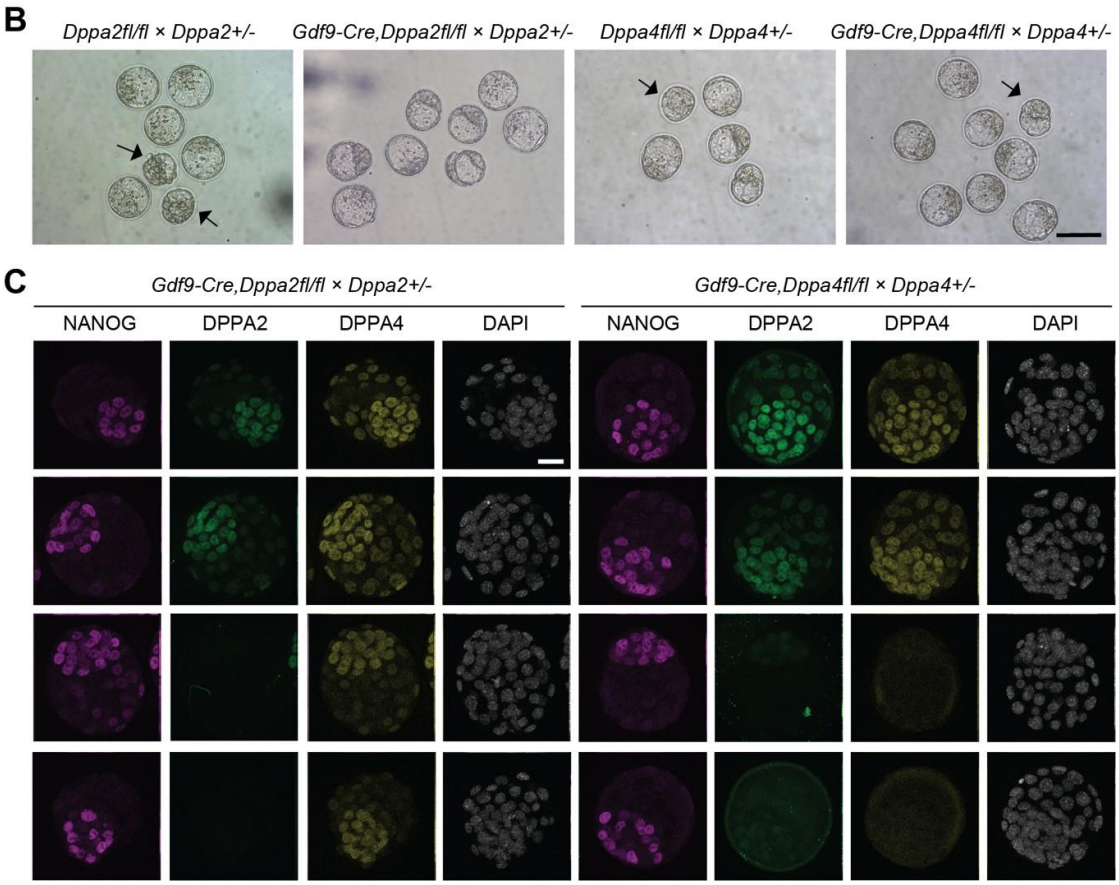

Fig. R1. Analysis of *in vivo* blastocysts collected after natural mating.
A) A table summarizing the *in vivo* blastocysts collected and analyzed. *Dppa2* or *Dppa4* maternal- zygotic knockout (*m-z-*) embryos were identified by immunostaining.

B) Representative images of blastocysts flushed from reproductive tracts after natural mating. Each image was obtained from one litter. Arrows point to not fully expanded blastocysts that were found in both control and CKO groups. Scale bar: 80 μm.
C) Representative immunostaining images showing Dppa2/4 maternal knockout (m-z+, top two rows) and maternal-zygotic knockout (m-z-, bottom two rows) blastocysts. Scale bar: 20 μm.

Other issues that the authors may wish to address include:
Comment #3: Fig. 1C, 2D and 3A: Data from more embryos would provide more robust results.

Response: For Fig. 1C and 2D, there were 5-14 embryos analyzed per group, which we believe is sufficient to reach conclusive interpretations. For Fig. 3A, as suggested, we performed additional *in vitro* culture experiments and genotyped a subset of embryos by immunostaining. More detailed info can be found in our response to Comment #4 of this reviewer.

Comment #4: In Fig. 3A, the genotypes of single embryos in the m-z+/m-z- groups should be provided to confirm the expected Mendelian ratio of embryos at later stages (e.g., morula and blastocyst). Only if correct Mendelian ratios pertain can the authors conclude that Dppa2 or Dppa4 ablation does not affect pre- implantation development.

Response: We have performed additional *in vitro* culture experiments and genotyped a subset of blastocysts by immunostaining. We confirmed the expected ~50:50 Mendelian ratio of m-z+ and m-z- at this stage (**Fig. R2**). In corroboration with our *in vivo* blastocysts analyses (**Fig. R1**), Dppa2 or Dppa4 ablation does not affect preimplantation development. The newly generated data have been included in the revised manuscript (revised **Fig. 3A, 3B**, Line 156-157).

A *Dppa2/4 in vitro* blastocysts (96 hpf) summary

Mating pairs	# of embryos genotyped	m-z- (%)
Gdf9-Cre,Dppa2fl/fl × Dppa2+/-	18	8 (44.4)
Gdf9-Cre,Dppa4fl/fl × Dppa4+/-	40	18 (45.0)

B

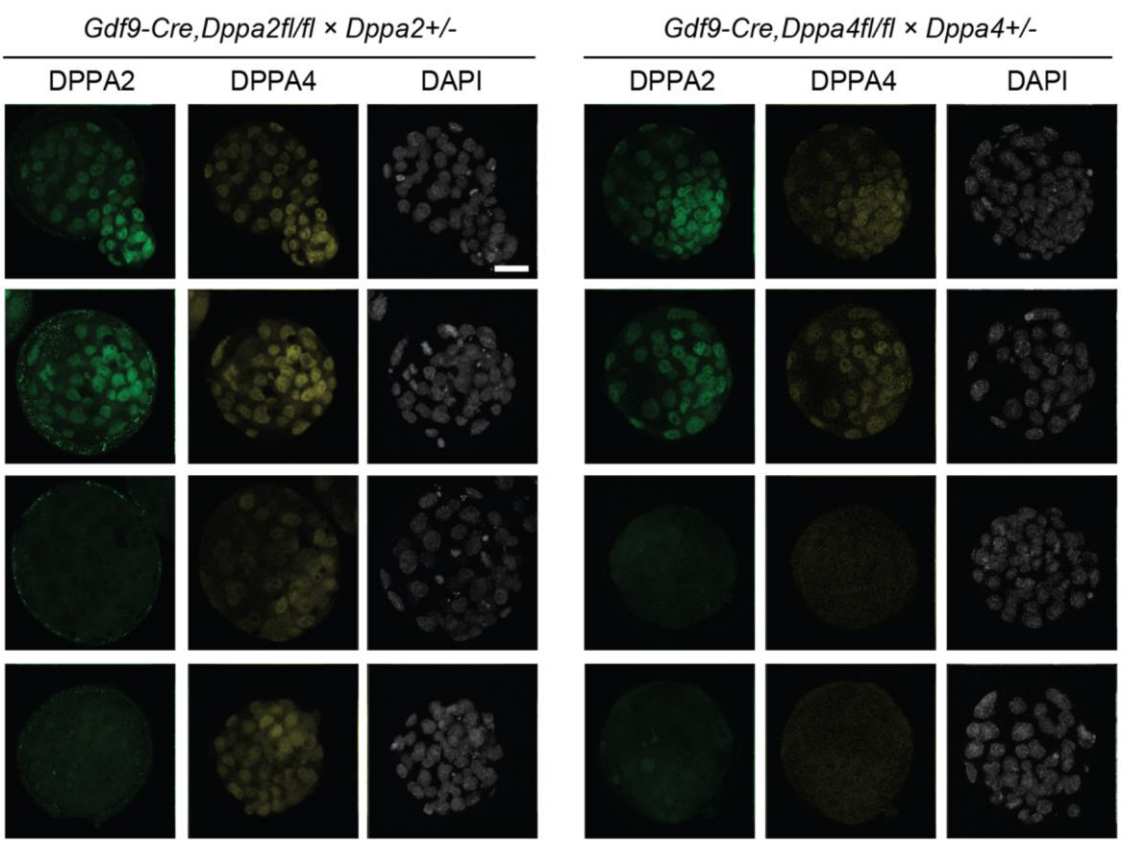

Fig. R2. Analysis of *in vitro* blastocysts.
A) A table summarizing the *in vitro* blastocysts genotyped by immunostaining.
B) Representative immunostaining images showing Dppa2/4 maternal knockout (m-z+, top two rows) and maternal-zygotic knockout (m-z-, bottom two rows) blastocysts. Scale bar: 20 μm.

Comment #5: The experiments in Fig. 3A should be repeated using natural mated females.

Response: We have addressed this comment in our response to Comment #2 of this reviewer.

Comment #6: Resolution of photos in Fig. 3B is poor: can better images be provided?

Response: We have replaced photos in original Fig. 3B (*in vitro* blastocysts) with pictures of *in vivo* blastocysts (**Fig. R1B**), which should be more conclusive. Thus, we have re-organized our Fig. 3 as shown in **Fig. R3.** Related texts have been modified accordingly (Line 156-161).

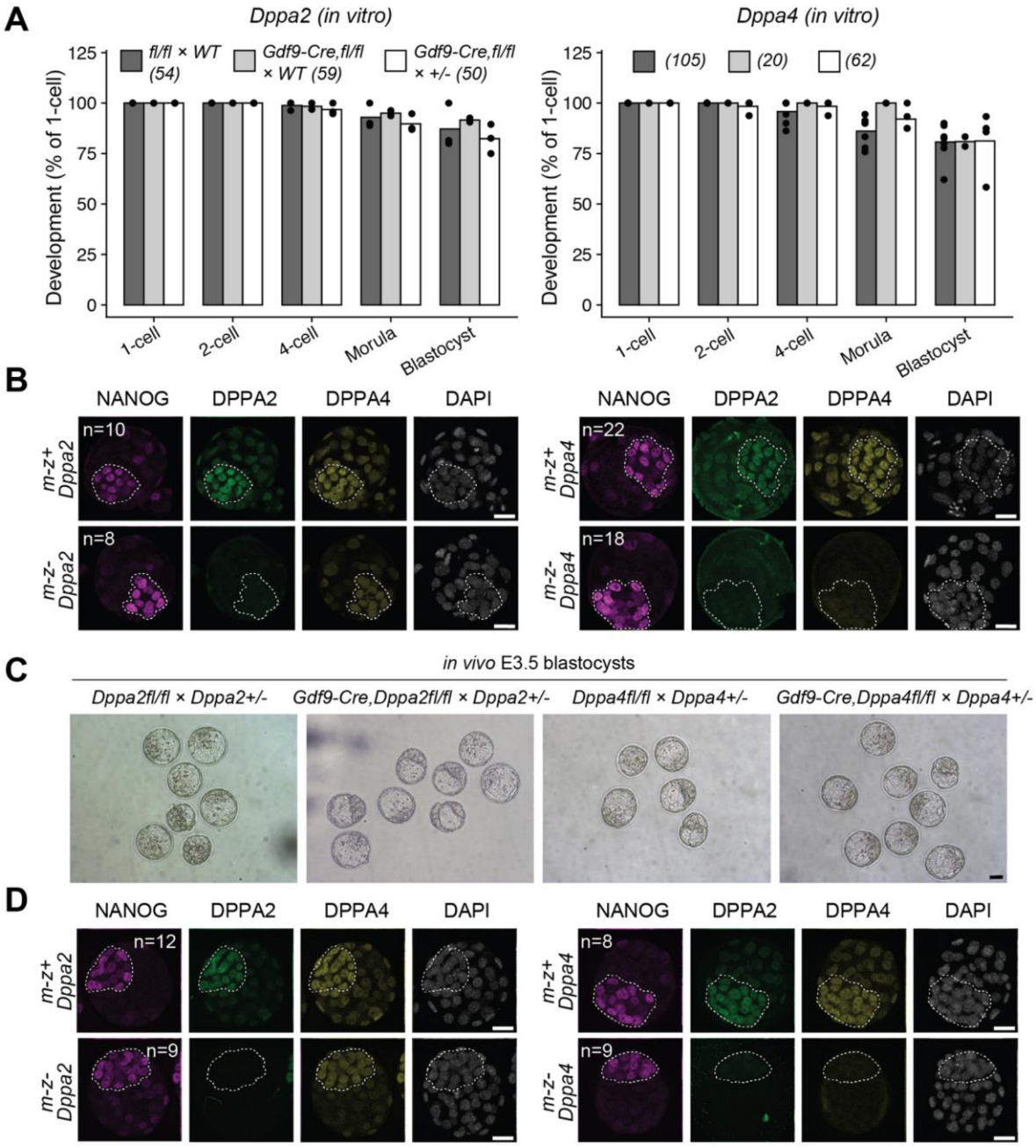

Fig. R3. Embryos without DPPA2 and DPPA4 undergo normal preimplantation development.
A) Bar graphs showing the percentage of embryos reaching the indicated developmental stages. The number of experiments performed are denoted by dots. Numbers of embryos analyzed for each group are as labeled. A subset of blastocysts from *Gdf9-Cre, fl/fl x +/-* were genotyped by immunostaining (panel **B**).
B) Images of *in vitro* blastocysts immunostained with antibodies against NANOG, DPPA2, DPPA4. The numbers of embryos with indicated genotypes are as shown. Scale bar: 20 μm.
C) Representative images of blastocysts flushed from reproductive tracts after natural mating. Each image was obtained from one litter. Scale bar: 20 μm.
D) Images of *in vivo* blastocysts immunostained with antibodies against NANOG, DPPA2, DPPA4. The numbers of embryos with indicated genotypes are as shown. Scale bar: 20 μm.

Comment #7: The litter size and genotypes of pups should be provided.

Response: We have provided relevant data as suggested. As shown in **Fig. R4,** although maternal Dppa2 or Dppa4 is not required for mouse development, few maternal-zygotic KO of Dppa2 or Dppa4 develop to adults (**Fig. R4A**). The lethality of m-z- mutants should occur around or after

birth because there was no or only slight Mendelian ratio distortions at E18.5 (**Fig. R4A**). However, the m-z- mutants already showed some phenotypic defects such as smaller sizes and/or pale skins by E18.5 (**Fig. R4B**). Thus, these observations suggest that loss of maternal-zygotic Dppa2 or Dppa4 causes peri-natal lethality, which is very similar to the previously reported zygotic *Dppa2/4* mutants (Madan et al., 2009; Nakamura et al., 2011). These results further support our initial conclusion that Dppa2/4 are not essential for mouse preimplantation development. These data have been included in the revised manuscript (Line 164-174, revised Fig. S2)

A

Dppa4 mating summary

Mating pairs	weaning pups /litters	litter size ± SD	m+z+ (%)	m-z+ (%)	m-z- (%)
Dppa4fl/fl × WT B6	55/9	6.9 ± 2.2	55 (100)	0 (0)	0 (0)
Gdf9-Cre,Dppa4fl/fl × WT B6	66/10	6.6 ± 2.2	0 (0)	66 (100)	0 (0)
Gdf9-Cre,Dppa4fl/fl × Dppa4+/-	64/14	4.5 ± 2.5	0 (0)	61 (95.3)	3 (4.7)

Mating pairs	E18.5 pups /litters	E18.5 pups ± SD	m+z+ (%)	m-z+ (%)	m-z- (%)
Gdf9-Cre,Dppa4fl/fl × Dppa4+/-	60/9	6.6 ± 1.2	0 (0)	34 (56.6)	26 (43.4)

Dppa2 mating summary

Mating pairs	weaning pups /litters	litter size ± SD	m+z+ (%)	m-z+ (%)	m-z- (%)
Dppa2fl/fl × WT B6	28/4	7.0 ± 1.8	28 (100)	0 (0)	0 (0)
Gdf9-Cre,Dppa2fl/fl × Dppa2+/-	15/3	5.0 ± 1.0	0 (0)	15 (100)	0 (0)

Mating pairs	E18.5 pups /litters	E18.5 pups ± SD	m+z+ (%)	m-z+ (%)	m-z- (%)
Gdf9-Cre,Dppa2fl/fl × Dppa2+/-	13/2	6.5 ± 3.5	0 (0)	5 (38.5)	8 (61.5)

B

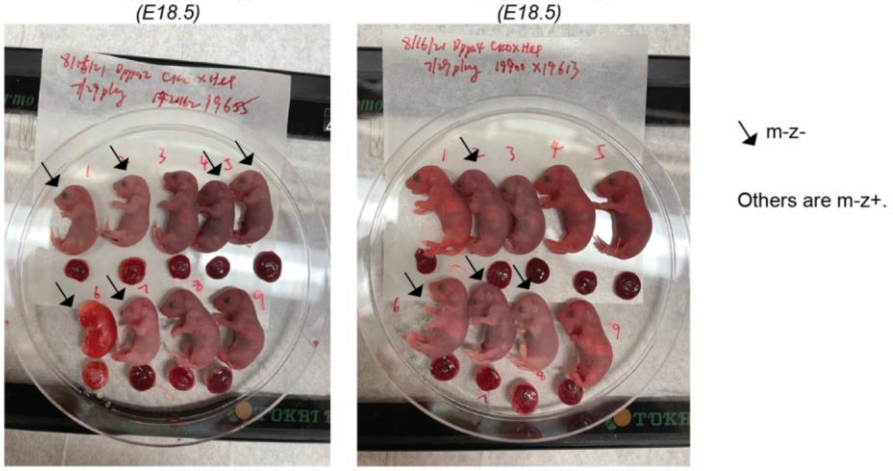

Fig. R4. Embryos without DPPA2 or DPPA4 undergo peri-natal lethality.
A) Mating summary of *Dppa2* and *Dppa4* mutants.
B). Representative images of E18.5 pups of *Dppa2* and *Dppa4* mutants.

Comment #8: In Fig. 4A, do all genomic views should use the same scale on the y axis? If a different scale is used, please clarify in figure or legend.

Response: All genomic views of *Dppa2* or *Dppa4 loci* used the same scale for Y axis. This info was included in the figure (next to the gene names on top)

Comment #9: Clarify what is meant by 'repeat' in the Fig. 4B legend and text. Why are there fewer datapoints in the repeat plots? Also, why not show all the genes listed in Fig. 4B in all 4 panels?

Response: "Repeat" here refers to transposable elements. As transposable elements have multiple insertions in the genome, the expression level of each repeat element is measured by the combined abundance of all insertions. Each data point represents one repeat element. Based on RepeatMasker, 1,245 repeat elements (e.g.., L1Md_A, MT2_Mm) are annotated in the mouse reference genome mm10, which explains why fewer datapoints in the repeat plots than the gene plots (46,078 annotated genes in mm10) and why genes such as Zscan4 cannot be shown in the repeat plots. We have included additional explanations in the methods section to avoid potential confusion (Line 284-292).

Comment #10: Same question (#9) for Fig. S3C.

Response: Please see our response to Comment #9.

Comment #11: In Fig. 4D, why not provide heatmap of more genes since the whole transcriptomes are sequenced? Results from more genes would make the conclusion more convincing.

Response: As the reviewer suggested, we have re-analyzed the public datasets and identified 170 ZGA genes that were down-regulated (fold change > 2 and adjusted p-value < 0.05) in either Dppa2 or Dppa4 KO ESCs (Eckersley-Maslin et al., 2019). As shown in **Fig. R5**, unlike in ESCs, activation of these genes in late 2-cell embryos are largely independent of Dppa2 or Dppa4, which are consistent with our original conclusion.

We still prefer our initial heatmap because it illustrates the well-known 2C genes and more directly delivers the message that 2C genes are normally activated in embryos without Dppa2 or Dppa4. In addition, we have also included the newly generated heatmap in **Fig. S4** of the revised manuscript.

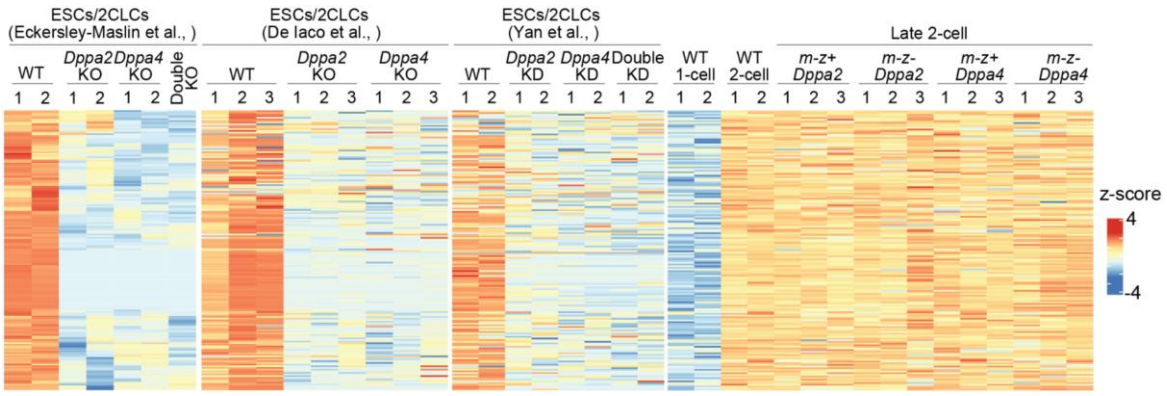

Fig. R5. Heatmap illustrating the expression levels of Dppa2/4-dependent ZGA genes in ESCs/2CLCs and 2-cell embryos. ZGA genes that were down-regulated in either Dppa2 or Dppa4 KO ESCs (fold change > 2 & adjusted p-value < 0.05) were selected (n = 170).

Comment #12: MuERV-L-Gag is widely used a marker for ZGA. Immunostaining of MuERV-L-Gag and/or other ZGA markers in WT embryo or embryos with Dppa2/4 depletion would provide more direct evidence for Dppa2/4 functions in ZGA and should be included in Fig. 4.

Response: We agree that MuERV-L-Gag is a marker for ZGA. However, we believe that immunostaining of MuERV-L-Gag does not add much to our data as we have used total RNA-seq, a more comprehensive technique than immunostaining, to quantify all ZGA genes.

Comment #13: Are ERCC spike-ins used for the RNA-seq experiments? If so, are they used for normalization of gene expression in different samples? Please provide more detail in "RNA-Seq libraries preparation and data processing"of Methods

Response: We did not include ERCC spike-in during library construction. We have expanded our Material & Methods section to avoid potential confusions.

Reviewer 2 Advance Summary and Potential Significance to Field:
In this manuscript, Chen et al. explore the potential role of DPPA2 and DPPA4 during preimplantation mouse development. They first summarise RNA expression from the literature and characterise protein distribution of both factors using immunohistochemistry in early embryos. Gdf9-Cre was employed to enable recombination of LoxP sites in either gene during early oocyte development: Dppa4 targeting was obtained courtesy of the authors of a previously published study, but Dppa2 fl/fl was generated and validated de novo by the present authors, adding a novel mouse line for the community. The mating scheme used to produce the compound deletion and control embryos is clearly presented. Quite surprisingly, and of interest to the field, the authors found that there was little effect of mutation of either DPPA2 or 4 on preimplantation mouse development. They confirm using immunohistochemistry the previous assumption, based upon the known operation of DPPA2 and 4 as heterodimers, that loss of one protein causes reciprocal reduction of the other, and thereby conclude that neither DPPA2 nor DPPA4 is required for early mouse development. This is an important finding because previous published studies using embryonic stem cells as a proxy for the embryo made the opposite assumption. We can conclude that, particularly in the context of cleavage stages of mammalian development, ESCs are not a good model, even if they can be shown to self-organise into structures resembling blastocysts.

Reviewer 2 Comments for the Author:
Comment #1: The materials and methods section and figure legends are very brief and more information should be provided. For example, how were the embryos in Fig.3B cultured? Were those embryos used for the IHC in Fig.3C? If not, were they flushed as blastocysts or cultured from earlier stages? The reader is unlikely to find out this information from the papers quoted in the methods, so a more detailed description is needed.

Response: Reviewer #1 also raised similar question. The embryos were cultured in KSOM medium and a subset of them were used for IHC analyses. We have also included in vivo blastocysts after natural mating and performed related IHC analyes. Additional details can be found in our responses to Comment #2, #4, and #6 of Reviewer #1.

Comment #2: In Fig.3B, could the authors explain why the m-z+ and m-z- embryos pooled?

Response: In this experiment, *Dppa2 or Dppa4* conditional KO oocytes were fertilized with heterozygotes (+/-) sperm cells. Thus, half of the embryos should be m-z+, whereas the other half should be m-z-. We have updated labels so that they can reveal such mating schemes in revised **Fig. 3A** (also in **Fig. R3A**) to avoid potential confusions.

Note to the authors for future work:
The use of tail tips for genotyping is generally discouraged these days, since the optimisation of PCR genotyping techniques means that a small ear biopsy provides ample material and is less invasive to the animal.

Response: We thank this reviewer for the suggestion and we will use ear biopsy for genotyping in the future.

Reviewer 3 Advance Summary and Potential Significance to Field:
Which maternal factors initiate zygotic genome activation (ZGA) remains a critical question for preimplantation development. Recently, 2C-like cells have emerged as a novel model to study ZGA. A novel TF that mediates ZGA, Dux, is identified through this model. However, Dux is not a maternal factor, and the maternal factors that activate Dux remain identified. Through the 2C-like cell model, Dppa2/4 has been identified as the transcription factor that activates Dux in mESCs. In addition, several studies provide evidence suggesting that Dppa2/4 plays a role in preimplantation development. All these indicate that Dppa2/4 is a maternal factor contributing to ZGA. Nevertheless, whether Dppa2/4 are crucial in ZGA has not been rigorously examined and remains an important question in the preimplantation development study.

Reviewer 3 Comments for the Author:
In this manuscript, Chen et al. provide the first evidence proving that Dppa2/4 are dispensable for ZGA. Their results suggest that Dppa2/4 is barely present in embryos during ZGA. The absence of Dppa2/4 shows minimum effects on the activation of ZGA genes, including Dux and transcripts activated by Dux. These results are unexpected but provide a piece of crucial information to the preimplantation development field. The data presented in this manuscript are concrete, well-organized, and sufficient to support their statements. I only have some minor comments.

Comment #1: Dppa2/4 shapes the DNA methylome of mESCs (Eckersley-Maslin, 2020; Eckersley-Maslin et al., 2020; Gretarsson and Hackett, 2020). Although Dppa2/4 is not present after implantation, the KO of Dppa2/4 impairs the post-implantation development, indicating that Dppa2/4 KO causes epigenetic alterations in blastocysts. Therefore, it would be nice should the authors provide the DNA methylome alterations in Dppa2/4 KO blastocysts and speculate how these alterations impairs embryonic development.

Response: We agree with the reviewer's interpretation. Our results indicate that Dppa2 and Dppa4 are dispensable for mouse preimplantation development but are required for proper post-implantation embryogenesis (**Fig. R4**). One likely mechanism is that Dppa2/4 safeguard bivalent genes from abnormal DNA methylation during the genome-wide wave of *de novo* DNA methylation around E5.0-E6.5. Indeed, we have performed some preliminary analyses showing that loss of Dppa2/4 causes ectopic DNA methylation at some bivalent gene promoters in E6.5 epiblast. However, given that the focus of this work is to investigate the fuction of Dppa2/4 in ZGA and preimplantation development, the related DNA methylome analyses are out the scope of the current study.

2. Several previous studies (Hu et al. 2010; Yan et al. 2019) suggest that Dppa2/4 are functionally important for ZGA. Thus, I recommend that the authors provide a detailed discussion on the inconsistency between their results and previous studies.

Response: These earlier studies investigated the dominant negative effects of Dppa2 mutants in early embryos. Overexpression of Dppa2 lacking the DNA-binding domain (Hu et al., 2010) or a sumoylated Dppa2 (Yan et al., 2019) impairs preimplantation development. However, the phenotypic defects could be due to some unknown side effects of RNA overexpression. In fact, overexpression of WT Dppa2 or Dppa4 causes 1- cell arrest of cloned embryos (Yang et al., 2020). Thus, our Cre-loxp approach is more convincing on the dispensable role of Dppa2/4 in mouse preimplantation development. We have included relevant discussions in the revised manuscript (Line 85-91).

References:

Eckersley-Maslin, M., Alda-Catalinas, C., Blotenburg, M., Kreibich, E., Krueger, C., and Reik, W. (2019). Dppa2 and Dppa4 directly regulate the Dux-driven zygotic transcriptional program. Genes & development *33*, 194-208.

Gretarsson, K.H., and Hackett, J.A. (2020). Dppa2 and Dppa4 counteract de novo methylation to establish a permissive epigenome for development. Nature structural & molecular biology *27*, 706-716.

Hernandez, C., Wang, Z., Ramazanov, B., Tang, Y., Mehta, S., Dambrot, C., Lee, Y.W., Tessema, K., Kumar, I., Astudillo, M., *et al.* (2018). Dppa2/4 Facilitate Epigenetic Remodeling during Reprogramming to Pluripotency. Cell stem cell *23*, 396-411 e398.

Madan, B., Madan, V., Weber, O., Tropel, P., Blum, C., Kieffer, E., Viville, S., and Fehling, H.J. (2009). The pluripotency-associated gene Dppa4 is dispensable for embryonic stem cell identity and germ cell development but essential for embryogenesis. Molecular and cellular biology *29*, 3186-3203.

Nakamura, T., Nakagawa, M., Ichisaka, T., Shiota, A., and Yamanaka, S. (2011). Essential roles of ECAT15- 2/Dppa2 in functional lung development. Molecular and cellular biology *31*, 4366-4378.

Yan, Y.L., Zhang, C., Hao, J., Wang, X.L., Ming, J., Mi, L., Na, J., Hu, X., and Wang, Y. (2019). DPPA2/4 and SUMO E3 ligase PIAS4 opposingly regulate zygotic transcriptional program. PLoS biology *17*, e3000324.

Yang, L., Liu, X., Song, L., Di, A., Su, G., Bai, C., Wei, Z., and Li, G. (2020). Transient Dux expression facilitates nuclear transfer and induced pluripotent stem cell reprogramming. EMBO Rep *21*, e50054.

Second decision letter

MS ID#: DEVELOP/2021/200178

MS TITLE: DPPA2 and DPPA4 are dispensable for mouse zygotic genome activation and preimplantation development

AUTHORS: Zhiyuan Chen, Zhenfei Xie, and Yi Zhang
ARTICLE TYPE: Research Report

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks. Where referee report on this version is available, it is appended below together with the Editor's note.

Reviewer 3

Advance summary and potential significance to field

In this study, the authors generated maternal KO and maternal-zygotic KO mouse embryos for Dppa2 and Dppa4, and determined their functions in Dux activation ZGA, and preimplantation embryo development. The results showed that Dppa2/4 are dispensable for ZGA and preimplantation development. Therefore, DUX and its downstream genes might play a key role in in vitro cultured embryonic stem cells, but their functions are not essential for in vivo development of early mouse embryos.
These findings are original and potentially have wide impacts in the field of developmental biology and stem cell studies.

Heng-Yu Fan

Comments for the author

In my opinion, the manuscript is appropriately revised, and all reviewer's comments are sufficiently addressed.