The lysine deacetylase activity of histone deacetylases 1 and 2 is required to safeguard zygotic genome activation in mice and cattle

Yanna Dang, Shuang Li, Panpan Zhao, Lieying Xiao, Lefeng Wang, Yan Shi, Lei Luo, Shaohua Wang, Huanan Wang and Kun Zhang
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MS TITLE: The histone deacetylase activity of HDAC1/2 is required to safeguard zygotic genome activation in mice and cattle

AUTHORS: Yanna Dang, Shuang Li, Panpan Zhao, Lieying Xiao, Lefeng Wang, Yan Shi, Lei Luo, Shaohua Wang, Huanan Wang, and Kun Zhang

Dear Dr. 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 considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. In particular, the reviewers are concerned about some overstatements regarding the specificity of the manipulations performed, for example, conclusions about specific histone modifications in spite of the fact of the known non-specific way of action of HDACs. The reviewers, and the editors, also agree that some important controls and clarifications are missing in the manuscript. If you are able to revise the manuscript along the lines suggested, which require additional 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.

Maria Elena Torres-Padilla
Handling Editor
Development

Reviewer 1

Advance Summary and Potential Significance to Field

This manuscript by Dang and colleagues addresses the function of H3K27ac in mouse and bovine preimplantation development. To do so, they study Hdac1/2 function at ZGA in and find that expression of a catalytically inactive Hdac1 or 2 (H1/H2MU), but not wild-type Hdac leads to a 2-cell stage block, suggesting defects in ZGA. Indeed they find widespread defects in gene expression at the late 2-cell stage, the time of ZGA in mice. They suggest this function is conserved in cattle as inhibition of Hdac1/2 activity results in similar defects in bovine embryo ZGA and development. Previously it has been demonstrated that Hdac1 but not Hdac2 is important for ZGA and preimplantation development in mice. Here the authors go further by suggesting that the catalytic activity of Hdac1 is responsible for this phenotype, through the use of H1MU overexpression. They go on to demonstrate that H1MU expression leads to a failure of broad H3K4me3 domain reprogramming at ZGA in mice, and show that the phenotype of H1MU can be rescued by expression of a H3K4me3 demethylase, Kdm5b. Thus this manuscript in principle, significantly extends previous findings on the action of Hdac1 and histone acetylation during early development.

Comments for the author

I have some serious concerns over the experimental design and interpretation of the results, which should be addressed in order for the conclusions of this study to be fully supported.

Major
1. Hdacs are known to be promiscuous enzymes, with little specificity for particular acetylated lysines. Therefore it is not possible to conclude a specific role for a particular lysine acetylation, based on experiments with Hdacs. Thus the authors should tone down their conclusions on a role for H3K27ac in mouse preimplantation development or gene expression based on their experiments with HDAC1/2 expression or HDAC1/2 inhibitors. For example the sentences: "Overall, these data revealed that the acute removal of H3K27ac mediated by HDAC1/2 is crucial for ZGA in both mouse and bovine embryos" (page 7), and "Here, we demonstrated HDAC1/2-mediated removal of H3K27ac is critical for establishing correct gene expression profile during ZGA" (Discussion) must be modified to reflect this. The observed developmental effect could equally be attributed to other (histone) lysine acetylations and unless the authors address all other potential substrates of HDAC1/2 they cannot determine a functional role for H3K27ac specifically.

Correlations between the presence of H3K27ac and gene expression are not enough to conclude a specific role for H3K27ac in the observed gene expression changes, as most histone tail acetylations are correlated with transcription, while their causal role is not clear. Thus, sentences such as the below (line 250) should be modified accordingly:

"On the contrary, the promoters and gene bodies of down-regulated genes displayed reduced H3K27ac signal (Fig 4f and S5e). These results suggest that the disorder of gene expression pattern caused by H1MU is likely attributed to the aberrant H3K27ac distribution."

2. I have concerns over the experimental design, regarding the overexpression of mutant Hdac1 (H1MU). The authors use this mutant enzyme as a dominant negative to block endogenous Hdac1 activity and conclude that the catalytic activity of Hdac1 is important for the observed effects. However, overexpression of H1MU leads to increased H3K27ac compared to wild-type embryos at the 2-cell stage, thus not only blocking the Hdac1 activity in removing H3K27ac, suggesting indirect effects.

Previous research demonstrates an important role for Hdac1 in ZGA and embryonic development in mice using a knockdown approach (Ma et al., Dev. Biol. 2008), which should be referenced in their manuscript. However the phenotype of their dominant negative Hdac1 results in a significantly stronger phenotype (2-cell block) than Hdac1 knockdown (developmental delay). While this could be due to a number of reasons (e.g. insufficient knockdown, compensation from Hdac2/3 under knockdown conditions reported in that paper) it again cautions a non-specific effect of the H1MU.

In fact the authors report a failure to activate many ZGA genes in H1MU conditions, accompanied by decreased histone acetylation, which they attribute to indirect effects in the discussion. The occurrence of a strong phenotype with H2MU also suggests indirect effects as Hdac2 was previously reported to not be important for ZGA and development (Ma et al., Dev. Biol. 2008).

To more directly address the specific function of Hdac1 catalytic activity, knockdown experiments for Hdac1 should be performed and subsequent rescue experiments with siRNA resistant H1MU or H1WT.

3. It is unclear to me why the transcriptional analysis (all of Figure 3) was performed comparing H1MU to H1WT expressing conditions. In this case it is not possible to conclude which genes are misregulated compared to normal embryos, especially considering that neither condition has wild-type levels of H3K27ac. Comparisons should be performed to wild-type embryos (H20 condition).
Do the widespread transcriptional changes observed upon H1MU overexpression (Figure 3c) reflect a developmental delay, as suggested by the results in Figure 3d and e? To address this clustering analysis or principal component analysis based on RNA-seq data from wild-type zygote, early and late 2-cell stage along with H1MU-expressing late 2-cell stage embryos should be performed.
In Figure 5, data from wild-type embryos (H20 condition) should also be included as well as H1WT and H1MU expressing embryos. Are Dux, MervL and Zscan4 upregulated in H1MU embryos compared to normal embryos?

4. I don't see clear evidence that broad H3K4me3 domains are retained in H1MU embryos in the ChIP-seq data. Using established pipelines to identify broad H3K4me3 domains, how many domains are identified in wild-type 2-cell stage (H20) and how many broad domains are lost and gained in H1MU, compared to H20 conditions?

5. The rescue of the H1MU phenotype is a very interesting finding. They show that this overcomes the 2-cell block at least in a fraction of embryos. The authors write here a majority of embryos - whereas it looks like around 60% develop past 2-cell stage in Fig. 7d. In addition, the images in Fig. 7C show morula-stage embryos. Is Kdm5b expression able to rescue to development to the blastocyst stage or not? The text, abstract and conclusions should be modified to more accurately reflect the extent of the rescue.

6. Are H3K4me3 broad domains recovered to wild-type levels in H1MU + Kdm5b injected embryos? Images of H3K4me3 staining and respective quantification of wild-type embryos (H20 only injected) should be included in Fig7b.

7. The analysis of rescued genes after Kdm5b expression is seriously flawed in my view (Fig. 7e). According to the legend, full rescue refers to genes upregulated (log2 FC ? 0) comparing Kdm5b + H1MU vs H1WT. Why is the full rescue effect of Kdm5b in H1MU conditions compared to H1WT conditions? They should compare gene expression levels to wild-type embryos (H20 injected), by selecting differentially expressed genes from Fig 3 - compared to H20 - injected embryos, not H1WT embryos) and determining which percentage of those genes are still

differentially expressed or not after H1MU + Kdm5b injection, compared again to H20 injected (using the same thresholding as applied in Fig. 3).

The authors conclude that the expression of Dux and Zscan4 drop to normal levels after Kdm5b expression. However 'normal levels' i.e. wild-type levels, are not shown in Figures 7g and h.

Minor:

Late 2-cell stage should be added to the following sentence: "Furthermore, H3K4me3 signal can be barely seen in control embryos while the intensity was obviously increased in H1MU embryos, suggesting the removal of broad H3K4me3 domain is blocked"

Please check the Figure reference in the following: "Kdm5b is zygotic transcribed from early to late-2 cell stage (Fig S7g) while H3K27ac is accumulated at Kdm5b."

Add Figure references to the following: "In contrast, H1MU caused a reduction in H3K27ac enrichment at Kdm5b, suggesting H3K27ac regulates transcription of Kdm5b."

At what stage was the RNA-seq experiment performed after the Kdm5b rescue experiment?

Statistical testing should be performed for Fig. 5b and e, 6h, 7g and h and 8d.

Reviewer 2

Advance Summary and Potential Significance to Field

I greatly appreciated reading this manuscript by Yanna Dang and collaborators investigating the role of histone deacetylase activity of HDAC1/2 in mice and cattle preimplantation development. The authors used two inhibitory approaches, although only the pharmacological one was used in mouse. The results obtained are clear and show that HDAC1/2 is a critical histone modifier that participates in the regulation of ZGA.

Comments for the author

I have only a few comments that should however be addressed before publication in my opinion.
- Some important papers previously published on HDACs and on H3K27 acetylation are not mentioned: 1) the paper by Ma & Schultz 2008 (10.1016/j.ydbio.2008.04.011) showing that HDAC1 expression inversely correlates with H4K5ac during mouse development and that HDAC1 knockdown, but does result in elevated levels of expression of several ZGA genes in late 2-cell embryos - it would be important to compare this list of genes with the genes identified in the present study. 2) the paper by Hayashi-Takanaka et al. 2011 (doi:10.1093/nar/gkr343) who monitored H3K9ac and H3K27ac in mouse embryos produced either by IVF or somatic cell nuclear transfer, showing that a high level of H3K27 acetylation is important for normal embryo development.
- I have another main concern regarding the use of FK228 as a specific inhibitor of HDAC1/2. Is this inhibitor enough to conclude that "HDAC1 has a conserved function in the regulation of gene expression pattern during ZGA " in bovine embryos? Although FK228 is sold by Selleckchem as a specific inhibitor, I found several papers that suggest FK228 (also called Romidepsin or Depsipeptide) might actually affect many intracellular processes for example by inducing DNA damages (10.1016/j.dnarep.2011.10.014 ; 10.2174/156800909787314039). It would be better to perform some mRNA microinjections in bovine embryos as for the mouse.

I also have some minor concerns
- The use of alpha-amanitin on embryos to show that it affects acetylation is not worth it (fig 2A). This has already been demonstrated by Ma & Schultz 2008 (see above).
- Similarly, the immunostaining of Ser2P to evaluate RNA pol II activity is not very interesting compared to the RNAseq data.
- Could the authors mention the timing of mRNA microinjections? Stating that injections were performed at the "zygote" stage is not precise enough.

Reviewer 3

Advance Summary and Potential Significance to Field

In this manuscript, Dang et al. investigated the function of histone deacetylases (HDAC1 and HDAC2) in zygotic genome activation (ZGA) in mouse and cattle embryos. Immunofluorescence using an H3K27ac-specific antibody revealed the decrease of global H3K27ac levels during/after ZGA in both mouse and cattle embryos. Re-analysis of RNA-seq data also indicated the loss of H3K27ac at the transcription start sites (TSSs) of maternal genes. As the levels of HDAC1 and HDAC2 were increased after ZGA, these HDACs could mediate H3K27 deacetylation. An overexpression of HDAC1 and HDAC2 induced additional slight decreases of H3K27ac levels. More strikingly, the expression of the deacetylase catalytic mutants increased H3K27ac levels and the mRNA-injected embryos arrested at the 2-cell stage. RNA-seq and ChIP-seq using embryos expressing HDAC mutants confirmed the changes in transcripts and H3K27ac at the TSSs of ZGA-related genes. The H3K27ac-increased regions by HDAC mutant expression contained DUX binding motifs. HDAC1 mutant expression also disturbed H3K4me3 through dysregulation of Kdm5b. Some key results obtained by mouse embryos were confirmed using cattle embryos. Based on these data, the authors propose an essential function of HDAC1/2 in regulating gene activation and repression in ZGA.

Whereas epigenetic changes during ZGA have been reported, the regulatory mechanism remains largely unknown. This study shows evidence for HDAC1/2-mediated gene regulatory mechanism in ZGA, possibly conserved throughout mammals. I think this will provide a significant impact in the field of embryo development and maternal-to-zygotic transition.

Comments for the author

The manuscript is very well written in a logical order and the conclusions are well supported by the data.

I have only a few minor comments that may improve the manuscript before publication.

1. p7, line 173-176. The reason why the overexpression of H1MU inhibits H3K27ac deacetylation in the presence of the endogenous HDAC1 is not explained. I assume H3K27ac is blocked by H1MU. Clearly explain why the effect of H1MU is seen.

2. Supplementary Fig. 4. In (a), correlations between two biological replicates are shown. However, this should be presented as a (color-coded) correlation matrix so that the correlations between different samples are also evaluated in addition to those between replicates.

3. The decrease of H3K27ac levels in PN has been reported (PMID: 21576221; DOI: 10.1093/nar/gkr343), suggesting the presence of HDAC activity in PN. It may be interesting to integrate this observation in discussion (or at least cite in Introduction).

4. Some typos are found.
Double check if "s" is added to verbs with the third singular. e.g., line 273. a DNA-binding protein that recruit(s).
Line 290. H3K4me4 (H3K4me3)

First revision

Author response to reviewers' comments

We are very thankful to three anonymous referees and editors for their valuable comments and suggestions. We believe that the comments have been highly constructive and very useful to improve the quality of the revised manuscript. Thus, we have thoughtfully carried out revisions based on the comments and suggestions. Our point-by-point responses to the comments could be found as below. We hope that all these changes fulfill the requirements to make the manuscript acceptable for publication in Development. Please let us know if any further clarifications are necessary.

Point-by-point responses:
Reviewer 1 Advance Summary and Potential Significance to Field:
This manuscript by Dang and colleagues addresses the function of H3K27ac in mouse and bovine preimplantation development. To do so, they study Hdac1/2 function at ZGA in and find that expression of a catalytically inactive Hdac1 or 2 (H1/H2MU), but not wild-type Hdac leads to a 2-cell stage block, suggesting defects in ZGA. Indeed they find widespread defects in gene expression at the late 2-cell stage, the time of ZGA in mice. They suggest this function is conserved in cattle as inhibition of Hdac1/2 activity results in similar defects in bovine embryo ZGA and development. Previously it has been demonstrated that Hdac1 but not Hdac2 is important for ZGA and preimplantation development in mice. Here the authors go further by suggesting that the catalytic activity of Hdac1 is responsible for this phenotype, through the use of H1MU overexpression. They go on to demonstrate that H1MU expression leads to a failure of broad H3K4me3 domain reprogramming at ZGA in mice, and show that the phenotype of H1MU can be rescued by expression of a H3K4me3 demethylase, Kdm5b. Thus this manuscript in principle, significantly extends previous findings on the action of Hdac1 and histone acetylation during early development.
Response: We really appreciate the opportunity to revise our work and thank you for all the insightful comments and suggestions.

To our knowledge, there is no previous study demonstrating the important role of Hdac1 in ZGA. While we acknowledge that the previous study by Ma et al. (Ma and Schultz, 2008) indicates Hdac1 is important for preimplantation development, they present little evidence about Hdac1's role in ZGA. Indeed, Ma et al.'s study show that Hdac1 knockdown does not affect the development to morula stage (Fig. 5 in Ma et al.'s paper), much later than the stage when ZGA occurs in mice.

Reviewer 1 Comments for the Author:
I have some serious concerns over the experimental design and interpretation of the results, which should be addressed in order for the conclusions of this study to be fully supported.

Major
1. Hdacs are known to be promiscuous enzymes, with little specificity for particular acetylated lysines. Therefore it is not possible to conclude a specific role for a particular lysine acetylation, based on experiments with Hdacs. Thus the authors should tone down their conclusions on a role for H3K27ac in mouse preimplantation development or gene expression based on their experiments with HDAC1/2 expression or HDAC1/2 inhibitors. For example the sentences: "Overall, these data revealed that the acute removal of H3K27ac mediated by HDAC1/2 is crucial for ZGA in both mouse and bovine embryos" (page 7), and "Here, we demonstrated HDAC1/2-mediated removal of H3K27ac is critical for establishing correct gene expression profile during ZGA" (Discussion) must be modified to reflect this. The observed developmental effect could equally be attributed to other (histone) lysine acetylations and unless the authors address all other potential substrates of HDAC1/2 they cannot determine a functional role for H3K27ac specifically.
Response: Thanks for the constructive suggestion. The sentences have been revised as follows.

"Overall, these data revealed that the lysine deacetylase activity of HDAC1/2 is crucial for ZGA in both mouse and bovine embryos." "Here, we demonstrated the lysine deacetylase activity of HDAC1/2 is critical for establishing correct gene expression profile during ZGA."

Correlations between the presence of H3K27ac and gene expression are not enough to conclude a specific role for H3K27ac in the observed gene expression changes, as most histone tail acetylations are correlated with transcription, while their causal role is not clear. Thus, sentences such as the below (line 250) should be modified accordingly: "On the contrary, the promoters and gene bodies of down-regulated genes displayed reduced H3K27ac signal (Fig 4f and S5e). These results suggest that the disorder of gene expression pattern caused by H1MU is likely attributed to the aberrant H3K27ac distribution."

Response: Thanks for your insightful comments. The sentence has been revised as: "These results suggest that the disorder of gene expression pattern caused by H1MU is correlated with the aberrant H3K27ac distribution."

2. I have concerns over the experimental design, regarding the overexpression of mutant Hdac1 (H1MU). The authors use this mutant enzyme as a dominant negative to block endogenous Hdac1 activity and conclude that the catalytic activity of Hdac1 is important for the observed effects. However, overexpression of H1MU leads to increased H3K27ac compared to wild-type embryos at the 2-cell stage, thus not only blocking the Hdac1 activity in removing H3K27ac, suggesting indirect effects.

Response: We agree that this result suggests indirect effects. This effect could be explained by the fact that the state of histone acetylation is directly regulated by both histone acetylases and HDACs. It is reported that Dux can recruit CBP/P300 and enhance H3K27ac (Choi et al., 2016). Indeed, Dux is up-regulated in H1MU embryos (Fig. 5b). As a result, the increased H3K27ac observed in H1MU embryos could be partly attributed to the increased expression of Dux.

However, there are three evidences supporting direct effects of the catalytic activity of Hdac1 on phenotypes documented in the present study. First, H141A mutation used here have been well established as responsible for the lysine deacetylase activity of Hdac1 (Hassig et al., 1998; Mal et al., 2001; Qiu et al., 2011). Second, by comparing H1MU and H1WT groups, we can conclude that only a single mutation on the deacetylase activity results in observed effects since there is only one variable (the mutation) between H1MU and H1WT groups. Third, we have validated our experiments by using three independent batches of in vitro-transcribed RNA of H1MU and H1WT, and obtained similar results.

Previous research demonstrates an important role for Hdac1 in ZGA and embryonic development in mice using a knockdown approach (Ma et al., Dev. Biol. 2008), which should be referenced in their manuscript. However the phenotype of their dominant negative Hdac1 results in a significantly stronger phenotype (2-cell block) than Hdac1 knockdown (developmental delay). While this could be due to a number of reasons (e.g. insufficient knockdown, compensation from Hdac2/3 under knockdown conditions reported in that paper) it again cautions a non-specific effect of the H1MU.

Response: We are sorry for the mistake. We have added the reference as suggested.

In fact, we have also performed Hdac1/2 knockdown in order to determine their specific role in mouse preimplantation development (Zhao et al., 2020). In summary, we found that Hdac1 and 2 plays a redundant role in controlling morula-to-blastocyst transition rather than ZGA. We agree that there are a number of reasons explaining the different effects between the knockdown and dominant-negative approach used in the present study. One likely reason is that maternal Hdac1 and 2 protein cannot be efficiently inhibited by the knockdown approach evidenced by both Ma's and our study. Indeed, in Ma's paper, the Hdac1 exhibited no significant decrease in early 2-cell embryos of knockdown group (Fig. 4C in Ma et al.'s paper), and is reduced by only 55% in knockdown late 2-cell embryos (Fig. 7B in Ma et al.'s paper) compared to control. However, by using H1MU, we could acutely block the Hdac1's activity (Fig. 2).

In fact the authors report a failure to activate many ZGA genes in H1MU conditions, accompanied by decreased histone acetylation, which they attribute to indirect effects in the discussion. The occurrence of a strong phenotype with H2MU also suggests indirect effects as Hdac2 was previously reported to not be important for ZGA and development (Ma et al., Dev. Biol. 2008).

Response: It's premature to conclude Hdac2 is not important for ZGA based on Ma et al.'s study since Hdac2 could not be efficiently removed by using RNAi approach (Ma and Schultz, 2008). Indeed, as shown in Fig. 9C of Ma et al.'s paper (Ma and Schultz, 2008), the intensity of HDAC2

is not decreased significantly until 72h post injection of siRNA.

We believe the deacetylase activity of Hdac2 is important for ZGA due to following reasons. First, similar with H1MU experiments, we also set up both H2O and H2WT groups as control for H2MU and found H2MU caused a severe defect in ZGA. Second, we routinely perform mRNA microinjection into mouse zygotes and have found only H1MU and H2MU leads to 2 cell block. Third, we have also validated our experiments by using three independent batches of in vitro-transcribed RNA of H2MU and H2WT, and obtained similar results.

To more directly address the specific function of Hdac1 catalytic activity, knockdown experiments for Hdac1 should be performed and subsequent rescue experiments with siRNA resistant H1MU or H1WT.

Response: Thanks for the suggestion. In fact, we have performed similar experiments in our previous study (Zhao et al., 2020). We found double knockdown of Hdac1 and 2 (cKD) results in developmental arrest during morula to blastocyst transition and the development of cKD embryos could be rescued (blastocyst rate>70%) by co-injection of exogenous Hdac1 and/or Hdac2 mRNA transcribed in vitro that were not targeted by the siRNAs (Fig. R1; n = 3, P < 0.05; reproduced from Figure 1j and S4D in (Zhao et al., 2020)). However, H1MU does not rescue the development and even induces 2-cell block in knockdown group, a phenotype stronger than the knockdown group (Fig. R1c). This experiment suggests that the knockdown strategy masks the effect of Hdac1/2 in ZGA due to the presence of maternal Hdac1/2.

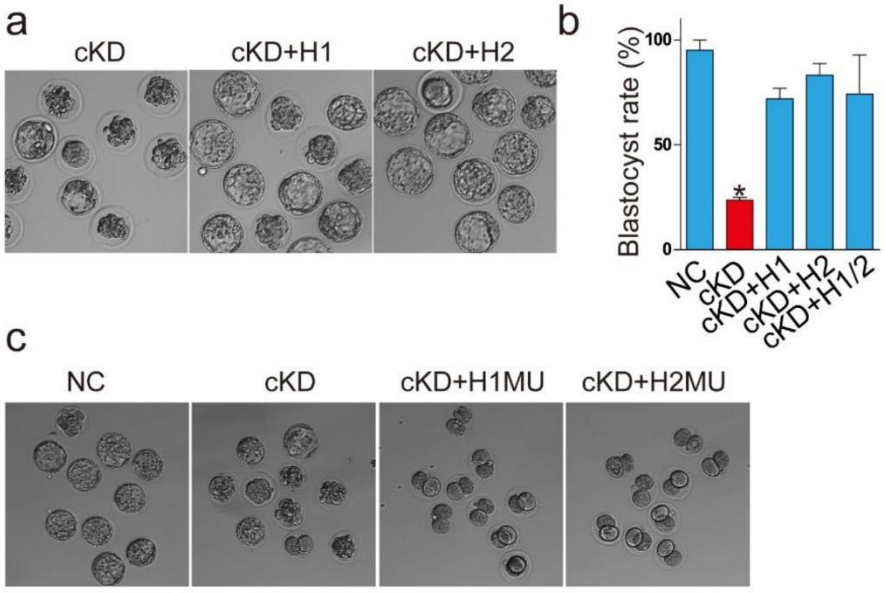

Fig. R1. Rescue of Hdac1 and 2 knockdown (cKD) embryos by microinjection of exogenous Hdac1 and/or Hdac2 mRNA (a and b; n = 3; *P < 0.05), but not by Hdac1 or Hdac2 mutant mRNA (c; n=2).

3. It is unclear to me why the transcriptional analysis (all of Figure 3) was performed comparing H1MU to H1WT expressing conditions. In this case it is not possible to conclude which genes are mis-regulated compared to normal embryos, especially considering that neither condition has wild-type levels of H3K27ac. Comparisons should be performed to wild-type embryos (H20 condition). Do the widespread transcriptional changes observed upon H1MU overexpression (Figure 3c) reflect a developmental delay, as suggested by the results in Figure 3d and e? To address this clustering analysis or principal component analysis based on RNA-seq data from wild- type zygote, early and late 2-cell stage along with H1MU-expressing late 2-cell stage embryos should be performed.

Response: Because the objective of the RNA-seq experiment was to determine the specific role of the deacetylase activity of Hdac1, thus we compared transcriptomes between H1MU and H1WT to rule out the possibility that other portion of Hdac1 affects gene expression. In fact, we

have also set up H2O groups and found no significant difference between H1WT and H2O either in phenotypes (Fig 2f) or transcriptomes (Fig R1a). Hence, similar results can be obtained either using H2O or H1WT as the control (Fig R1b).

As for the concern on the developmental delay, we performed hierarchical clustering of the RNA-seq data as suggested and found H1MU-expressing late 2-cell is not close to wild-type early 2-cell. Furthermore, RNA-seq analysis revealed that over 1800 developmental genes were prematurely expressed in H1MU late 2-cell embryos (cluster 3 in Fig. R2d and Fig. 3d), ruling out the possibility of developmental delay (Fig R2c, revised Fig S4e).

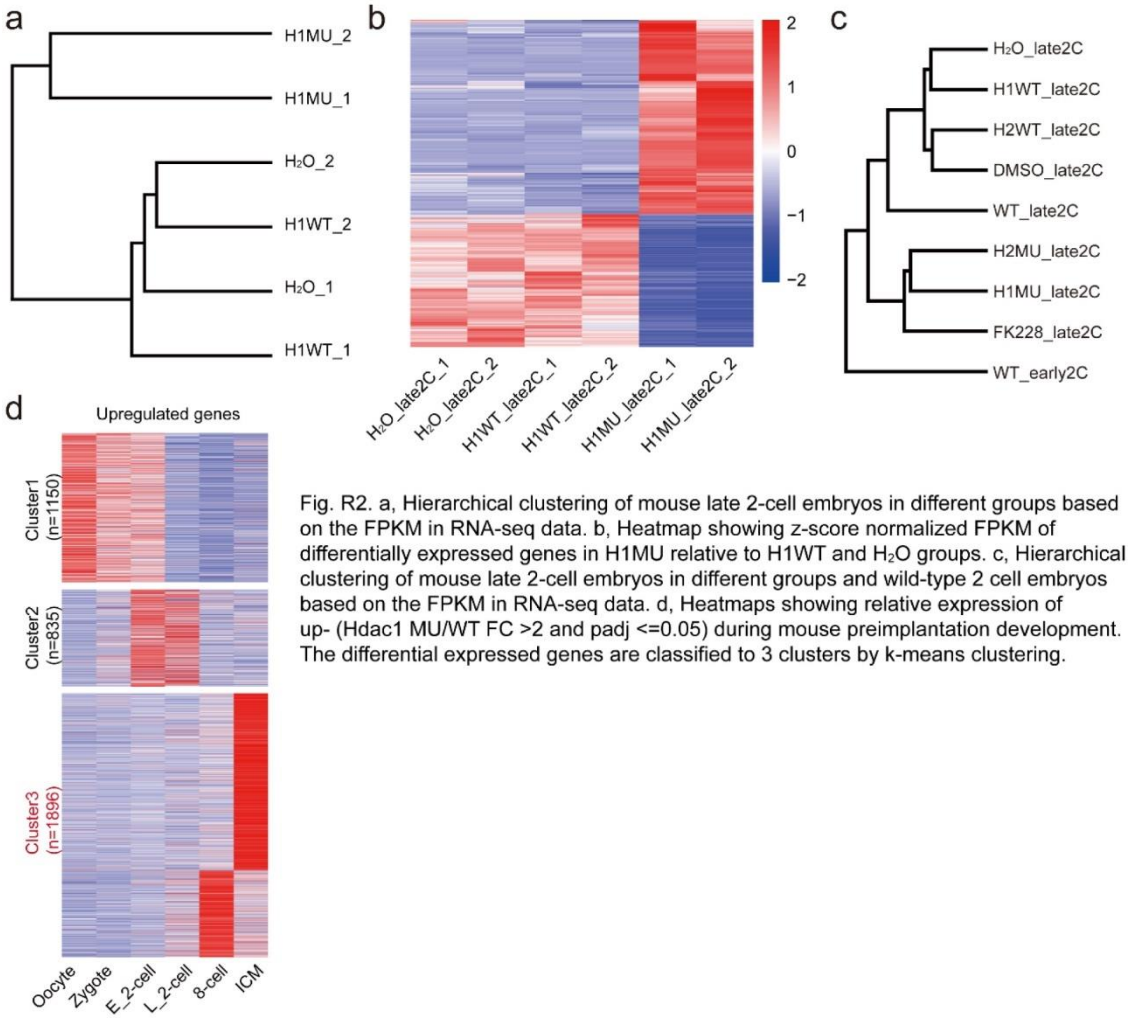

Fig. R2. a, Hierarchical clustering of mouse late 2-cell embryos in different groups based on the FPKM in RNA-seq data. b, Heatmap showing z-score normalized FPKM of differentially expressed genes in H1MU relative to H1WT and H_2O groups. c, Hierarchical clustering of mouse late 2-cell embryos in different groups and wild-type 2 cell embryos based on the FPKM in RNA-seq data. d, Heatmaps showing relative expression of up- (Hdac1 MU/WT FC >2 and padj <=0.05) during mouse preimplantation development. The differential expressed genes are classified to 3 clusters by k-means clustering.

In Figure 5, data from wild-type embryos (H20 condition) should also be included as well as H1WT and H1MU expressing embryos. Are Dux, MervL and Zscan4 upregulated in H1MU embryos compared to normal embryos?

Response: Thanks for your advice. Yes, *Dux*, *MervL*, *MT2* and *Zscan4* are also upregulated in H1MU embryos relative to normal embryos (Fig. R3). We have added the data into the corresponding figures (revised Fig 5b-e).

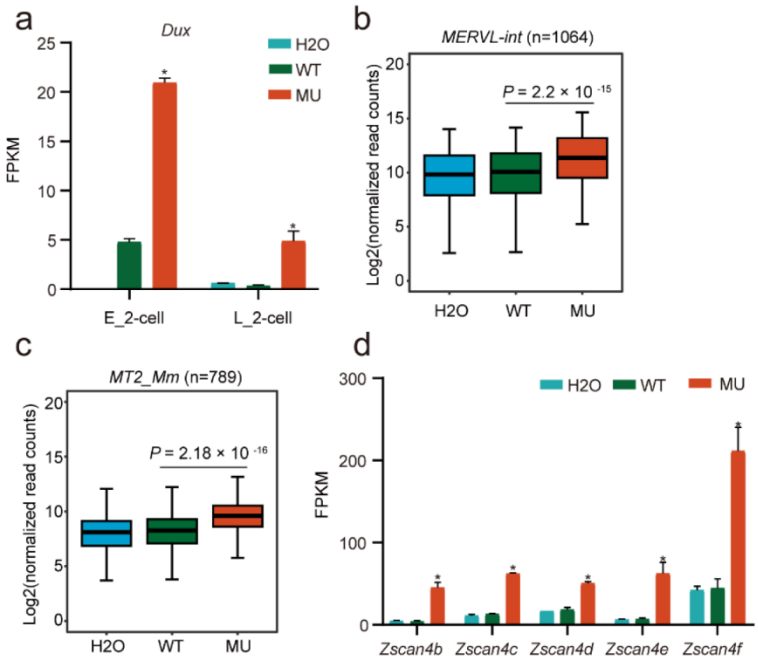

Fig. R3. Dux, MervL and Zscan4 are upregulated upon HDAC1 mutation.
a, Relative expression of Dux (also known as Duxf3) in early and late 2-cell embryos. b, c, Box plots showing expression of MERVL-int (n=1064) and MT2_Mm (n=789) in late 2-cell embryos. Read counts are normalized by DESeq2. P values are calculated by Wilcoxon rank sum test. d, Relative expression of Zscan4s in late 2-cell embryos.

4. I don't see clear evidence that broad H3K4me3 domains are retained in H1MU embryos in the ChIP-seq data. Using established pipelines to identify broad H3K4me3 domains, how many domains are identified in wild-type 2-cell stage (H20) and how many broad domains are lost and gained in H1MU, compared to H20 conditions? Response: Thank you for the reminder. To compare the changes of H3K4me3 in H1MU embryos, the mouse genome was scanned using a sliding window of 5 kb and step size of 1 kb, and then RPKM for each window was calculated. Next, H3K4me3 signals were compared between H2O and H1MU. The H3K4me3-gained or -lost regions were identified with following threshold: log2 (fold change) > 1.5 for gained regions or < - 1.5 for lost regions, and sum of RPKM in H2O and H1MU >1. Finally, we identified 34590 H3K4me3-gained domains and 5890 H3K4me3-lost domains in H1MU, which covered 4.5% and 0.7% of the genome respectively. Furthermore, nearly 50% of H3K4me3-gained domains were located in distal intergenic regions (Fig 6c), which should be referred as broad/non-canonical H3K4me3 domains. We have added these information into the revised manuscript.

5. The rescue of the H1MU phenotype is a very interesting finding. They show that this overcomes the 2-cell block at least in a fraction of embryos. The authors write here a majority of embryos – whereas it looks like around 60% develop past 2-cell stage in Fig. 7d. In addition, the images in Fig. 7C show morula-stage embryos. Is Kdm5b expression able to rescue to development to the blastocyst stage or not? The text, abstract and conclusions should be modified to more accurately reflect the extent of the rescue.
Response: Kdm5b expression did rescue the development of H1MU to past 2-cell stage with the majority of embryos develop to 4/8-cell and morula stage. However, Kdm5b expression was not able to rescue the development to blastocyst stage, suggesting an important role of Hdac1/2 during morula-to-blastocyst transition as we determined previously (Zhao et al., 2020). Thanks for your advice, and we have refined the description of this section in the revised version to reflect the extent of rescue in greater detail.

6. Are H3K4me3 broad domains recovered to wild-type levels in H1MU + Kdm5b injected embryos? Images of H3K4me3 staining and respective quantification of wild- type embryos (H20 only injected) should be included in Fig7b.

Response: Thanks for your advice. Although overexpression of Kdm5b decreased

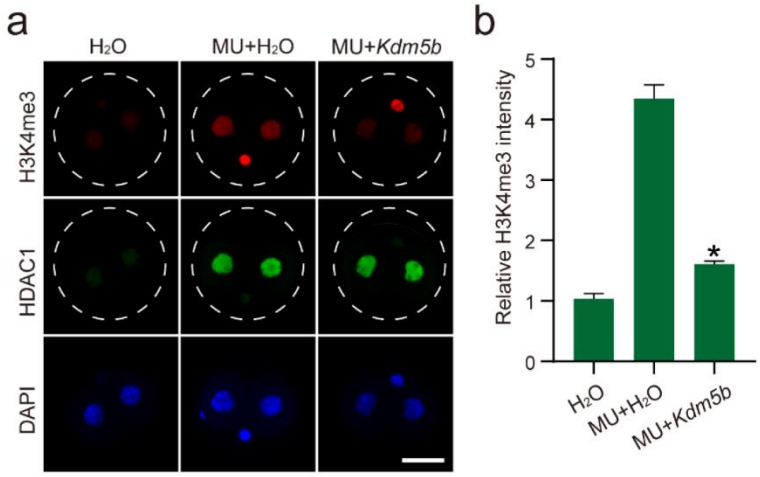

Fig. R4. Immunofluorescence staining of H3K4me3 and HDAC1 at late 2-cell stage. a, representative images, scale bar, 25 μm, b, H3K4me3 intensity relative to embryos injected with H_2O.

H3K4me3 greatly compared to H1MU, H3K4me3 intensity in H1MU+Kdm5b group was still slightly higher than wild-type (Fig. R4), likely due to the dosage issue. As suggested, we have added the H2O injected embryos in Fig7b.

7. The analysis of rescued genes after Kdm5b expression is seriously flawed in my view (Fig. 7e). According to the legend, full rescue refers to genes upregulated (log2 FC ≥ 0) comparing Kdm5b + H1MU vs H1WT. Why is the full rescue effect of Kdm5b in H1MU conditions compared to H1WT conditions? They should compare gene expression levels to wild-type embryos (H20 injected), by selecting differentially expressed genes from Fig 3 – compared to H20 – injected embryos, not H1WT embryos) and determining which percentage of those genes are still differentially expressed or not after H1MU + Kdm5b injection, compared again to H20 injected (using the same thresholding as applied in Fig. 3).

Response: Thanks for your advice. As addressed above, there is no significant difference on global gene expression levels between H1WT and H2O injected embryos. Similar results could be obtained by comparing with H2O injected embryos (Fig. R5).

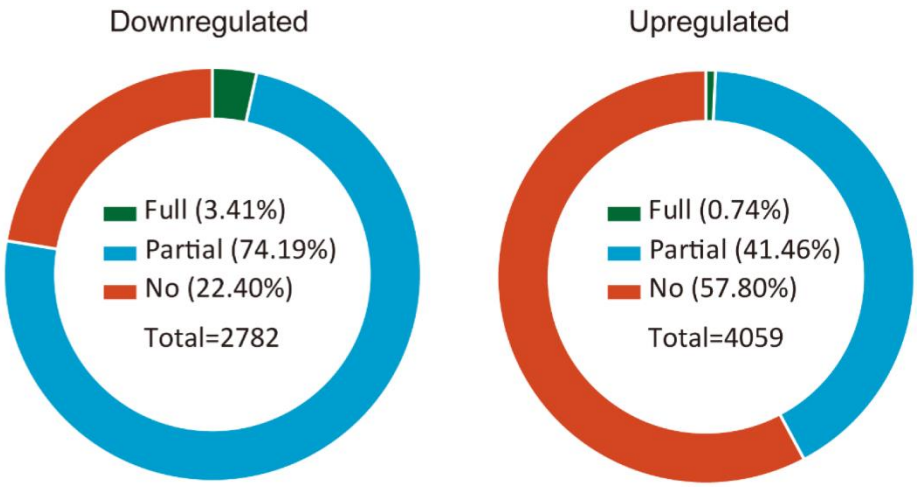

Fig. R5. Donut chart of down-regulated genes (H1MU/H_2O, left) and up-regulated genes (H1MU/H_2O, right) based on their extent of rescue in H1MU+Kdm5b mRNA injection embryos. For down-regulated genes, full rescue means genes characterized by a log2 ratio of H1MU+Kdm5b /H_2O≥0; partial rescue refers to genes characterized by a log2 ratio of H1MU+Kdm5b/H1MU>0; no rescue genes are characterized by a log2 ratio of H1MU+Kdm5b/H1MU≤0. For up-regulated genes, full rescue means genes characterized by a log2 ratio of H1MU+Kdm5b/H_2O≤0; partial rescue refers to genes characterized by a log2 ratio of H1MU+Kdm5b/H1MU<0; no rescue genes are characterized by a log2 ratio of H1MU+Kdm5b/H1MU≥0.

The authors conclude that the expression of Dux and Zscan4 drop to normal levels after Kdm5b expression. However 'normal levels' i.e. wild-type levels, are not shown in Figures 7g and h.

Response: Thanks for your reminder, we have added the data into Fig 7g and h.

Minor:
Late 2-cell stage should be added to the following sentence: "Furthermore, H3K4me3 signal can be barely seen in control embryos while the intensity was obviously increased in H1MU embryos, suggesting the removal of broad H3K4me3 domain is blocked"
Response: Thanks for your reminder, we have added the information into the corresponding sentence in the revised manuscript.

Please check the Figure reference in the following: "Kdm5b is zygotic transcribed from early to late-2 cell stage (Fig S7g) while H3K27ac is accumulated at Kdm5b." Response: Thanks for your reminder. We are so sorry for the mistake and have revised the corresponding figure reference.

Add Figure references to the following: "In contrast, H1MU caused a reduction in H3K27ac enrichment at Kdm5b, suggesting H3K27ac regulates transcription of Kdm5b."
Response: Thanks. We have added the figure references into the revised manuscript.

At what stage was the RNA-seq experiment performed after the Kdm5b rescue experiment?
Response: The RNA-seq experiment was performed at late 2-cell stage (Fig 7a).

Statistical testing should be performed for Fig. 5b and e, 6h, 7g and h and 8d.
Response: Thanks. We have performed statistical testing and revised the figures accordingly.

Reviewer 2 Advance Summary and Potential Significance to Field:
I greatly appreciated reading this manuscript by Yanna Dang and collaborators investigating the role of histone deacetylase activity of HDAC1/2 in mice and cattle preimplantation development. The authors used two inhibitory approaches, although only the pharmacological one was used in mouse. The results obtained are clear and show that HDAC1/2 is a critical histone modifier that participates in the regulation of ZGA.
Response: Thank you for your recognition of our work, which inspires us a lot.

Reviewer 2 Comments for the Author:
I have only a few comments that should however be addressed before publication in my opinion.
-Some important papers previously published on HDACs and on H3K27 acetylation are not mentioned: 1) the paper by Ma & Schultz 2008 (10.1016/j.ydbio.2008.04.011) showing that HDAC1 expression inversely correlates with H4K5ac during mouse development and that HDAC1 knockdown, but does result in elevated levels of expression of several ZGA genes in late 2-cell embryos – it would be important to compare this list of genes with the genes identified in the present study. 2) the paper by Hayashi-Takanaka et al. 2011 (doi:10.1093/nar/gkr343) who monitored H3K9ac and H3K27ac in mouse embryos produced either by IVF or somatic cell nuclear transfer, showing that a high level of H3K27 acetylation is important for normal embryo development.
Response: Thank you very much for taking time to evaluate our work.

Regarding paper 1, as suggested, we compare the list of differentially expressed genes in our study with those genes identified in Ma & Schultz's study (Fig. R6), and found that a couple of faithful Hdac1 targets (*Hdac2* and *p21/Cdkn1a*) were also increased in H1MU. However, other genes were down-regulated in H1MU. The difference could be attributed to the different experimental approaches used (RNAi approach used in Ma & Schultz's study vs Dominant negative approach in the present study).

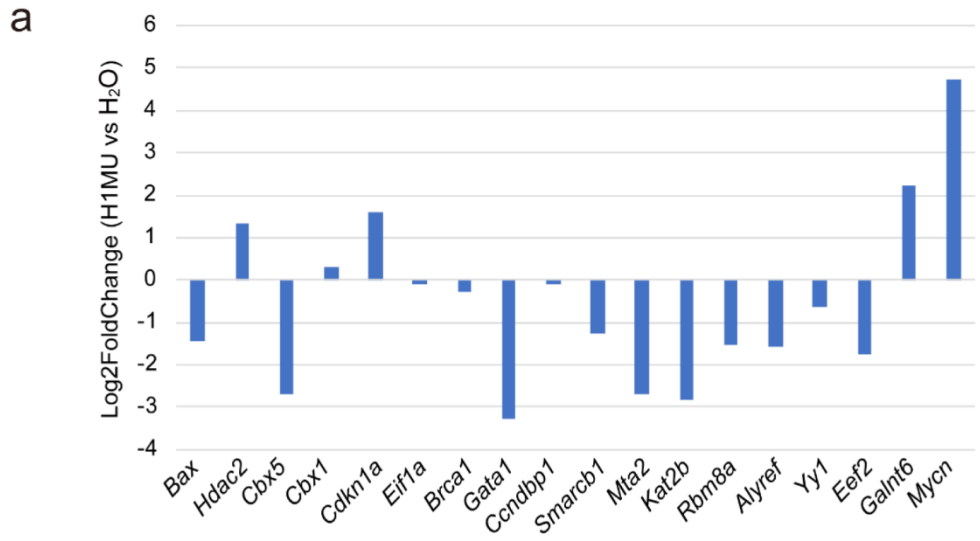

Fig.R6. Expression of those genes that examined in Ma & Schultz's paper in H1MU

Regarding paper 2, we agree that the study by Hayashi-Takanaka et al is very impressive and have added the reference into the manuscript.

-I have another main concern regarding the use of FK228 as a specific inhibitor of HDAC1/2. Is this inhibitor enough to conclude that "HDAC1 has a conserved function in the regulation of gene expression pattern during ZGA » in bovine embryos? Although FK228 is sold by Selleckchem as a specific inhibitor, I found several papers that suggest FK228 (also called Romidepsin or Depsipeptide) might actually affect many intracellular processes for example by inducing DNA damages (10.1016/j.dnarep.2011.10.014; 10.2174/156800909787314039). It would be better to perform some mRNA microinjections in bovine embryos as for the mouse.
Response: Thank you for the advice. There are two experiments supporting the specificity of FK228. First, our RNA-seq analysis in mouse embryos shows that 81.4% of differentially expressed genes induced by FK228 treatment overlapped with those caused by H1MU and H2MU (Fig. R7a). Second, We microinjected H1MU mRNA to bovine zygotes (Fig. R7b). Strikingly, we found similar results with FK228 experiment. Most of the H1MU embryos were arrested at 8/16-cell stage (Fig. R7c, R7d, Fig. S8d, e), with obviously increase of H3K27ac and H3K4me3 compared with H_2O injected embryos (Fig. R7e, R7f, Fig. S8f, g). These results further confirmed the importance of HDAC1 during ZGA in bovine embryos. We have included these results into the revised figure and manuscript.

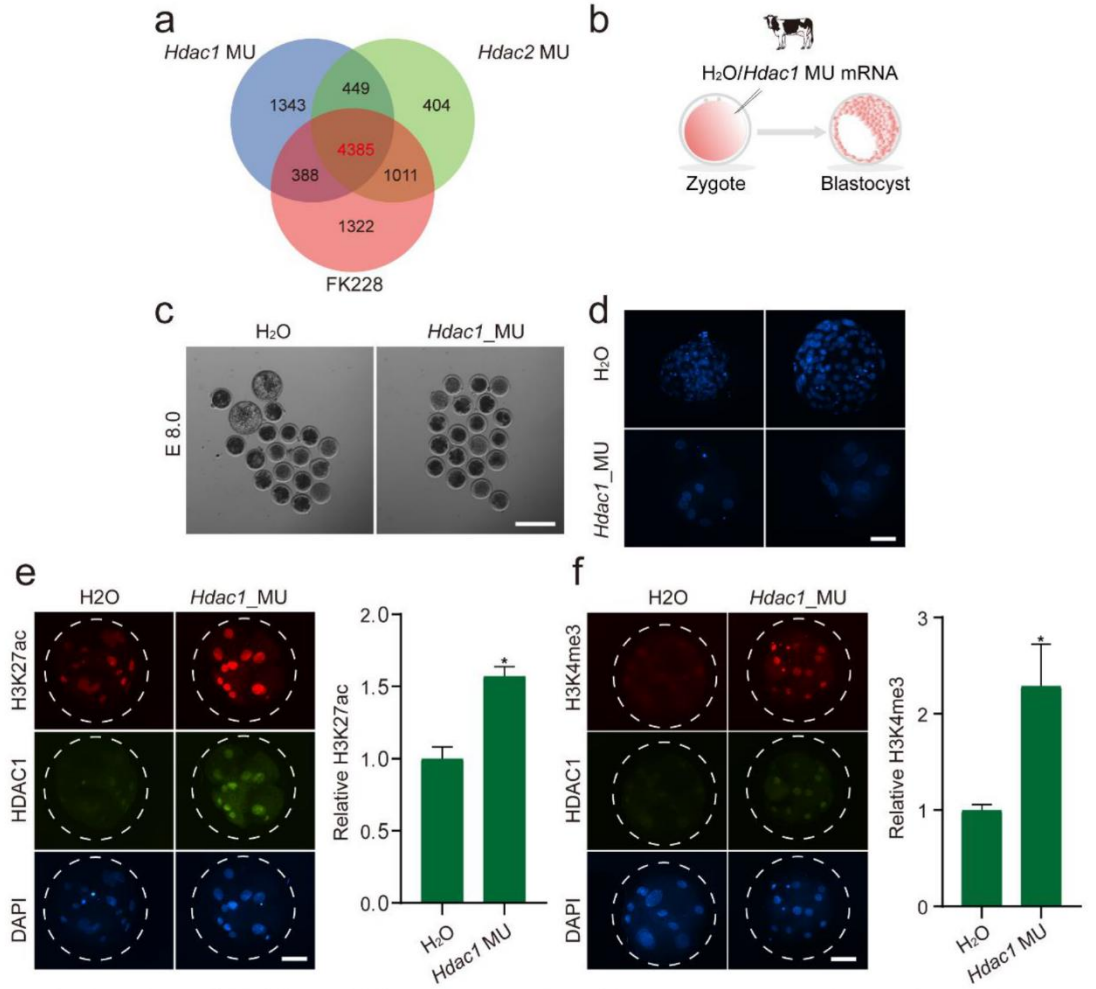

Fig. R7. Validation of FK228 specificity. a, overlap of all differentially expressed genes induced by overexpression of Hdac1 or Hdac2 MU mRNA and treatment of FK228. Size of the circles doesn't equal to the number of genes.Immunofluorescence staining of H3K4me3 and HDAC1 at late 2-cell stage. b, experimental scheme. c, representative images in bright field at day 7.5 after fertilization of bovine embryos injected with water or Hdac1 MU (n=4). d, DNA staining with DAPI at day 7.5 after fertilization. e, H3K27ac immunostaining at 8/16-cell stage, scale bar, 50 μm, and analysis. 10-15 embryos were analyzed per group. f, H3K4me3 immunostaining at 8/16-cell stage, scale bar, 50 μm, and analysis. 10-15 embryos were analyzed per group.

I also have some minor concerns
-The use of alpha-amanitin on embryos to show that it affects acetylation is not worth it (fig 2A). This has already been demonstrated by Ma & Schultz 2008 (see above).
Response: Thank you for the comment. We have double checked Ma's paper found no evidence showing alpha-amanitin's effects on acetylation.

-Similarly, the immunostaining of Ser2P to evaluate RNA pol II activity is not very interesting compared to the RNAseq data.
Response: Thanks for the comment, we have reduced the description of this result in the revised version.

-Could the authors mention the timing of mRNA microinjections? Stating that injections were performed at the "zygote" stage is not precise enough.
Response: The microinjection was performed 20~22 hrs post hCG injection, we have added the information into the Materials and Methods section.

Reviewer 3 Advance Summary and Potential Significance to Field:
In this manuscript, Dang et al. investigated the function of histone deacetylases (HDAC1 and HDAC2) in zygotic genome activation (ZGA) in mouse and cattle embryos. Immunofluorescence

using an H3K27ac-specific antibody revealed the decrease of global H3K27ac levels during/after ZGA in both mouse and cattle embryos. Re-analysis of RNA-seq data also indicated the loss of H3K27ac at the transcription start sites (TSSs) of maternal genes. As the levels of HDAC1 and HDAC2 were increased after ZGA, these HDACs could mediate H3K27 deacetylation. An overexpression of HDAC1 and HDAC2 induced additional slight decreases of H3K27ac levels. More strikingly, the expression of the deacetylase catalytic mutants increased H3K27ac levels and the mRNA-injected embryos arrested at the 2-cell stage. RNA-seq and ChIP-seq using embryos expressing HDAC mutants confirmed the changes in transcripts and H3K27ac at the TSSs of ZGA-related genes. The H3K27ac- increased regions by HDAC mutant expression contained DUX binding motifs. HDAC1 mutant expression also disturbed H3K4me3 through dysregulation of Kdm5b. Some key results obtained by mouse embryos were confirmed using cattle embryos. Based on these data, the authors propose an essential function of HDAC1/2 in regulating gene activation and repression in ZGA.
Whereas epigenetic changes during ZGA have been reported, the regulatory mechanism remains largely unknown. This study shows evidence for HDAC1/2-mediated gene regulatory mechanism in ZGA, possibly conserved throughout mammals. I think this will provide a significant impact in the field of embryo development and maternal-to- zygotic transition.
Response: Thank you very much for the recognition of our work.

Reviewer 3 Comments for the Author:
The manuscript is very well written in a logical order and the conclusions are well supported by the data.
I have only a few minor comments that may improve the manuscript before publication.
1. p7, line 173-176. The reason why the overexpression of H1MU inhibits H3K27ac deacetylation in the presence of the endogenous HDAC1 is not explained. I assume H3K27ac is blocked by H1MU. Clearly explain why the effect of H1MU is seen.
Response: Thanks for the comment. The mutant (H1MU) is designed to change histidine 141 to alanine (H141A), and abolish the deacetylase activity of the endogenous HDAC1 likely through competitive inhibition without destroying the interaction with HDAC1-associated proteins (Hassig et al., 1998). We have included the explanation for H1MU in the revised manuscript (Line 180-182)

2. Supplementary Fig. 4. In (a), correlations between two biological replicates are shown. However, this should be presented as a (color-coded) correlation matrix so that the correlations between different samples are also evaluated in addition to those between replicates.
Response: We agree with your insightful advice and have replaced the Fig S4a using heatmaps (Fig S4a).

3. The decrease of H3K27ac levels in PN has been reported (PMID: 21576221; DOI: 10.1093/nar/gkr343), suggesting the presence of HDAC activity in PN. It may be interesting to integrate this observation in discussion (or at least cite in Introduction).
Response: Thank you very much for reminding us this important work. The decrease of H3K27ac in PN further consolidates our conclusion that H3K27ac reprogramming is likely one upstream inducer of the major ZGA since it occurs prior to ZGA. We have integrated this study into the revised manuscript (Line 406).

4. Some typos are found.
Double check if “s” is added to verbs with the third singular. e.g., line 273. a DNA- binding protein that recruit(s).
Line 290. H3K4me4 (H3K4me3)
Response: We are so sorry for these mistakes. We have revised them carefully (Line 290 and 307).

References:
Choi, S.H., Gearhart, M.D., Cui, Z., Bosnakovski, D., Kim, M., Schennum, N., and Kyba, M. (2016). DUX4 recruits p300/CBP through its C-terminus and induces global H3K27 acetylation changes. Nucleic Acids Res *44*, 5161-5173.
Hassig, C.A., Tong, J.K., Fleischer, T.C., Owa, T., Grable, P.G., Ayer, D.E., and Schreiber, S.L. (1998). A role for histone deacetylase activity in HDAC1-mediated transcriptional repression. Proc Natl Acad Sci U S A *95*, 3519-3524.

Ma, P., and Schultz, R.M. (2008). Histone deacetylase 1 (HDAC1) regulates histone acetylation, development, and gene expression in preimplantation mouse embryos. Dev Biol *319*, 110-120. Mal, A., Sturniolo, M., Schiltz, R.L., Ghosh, M.K., and Harter, M.L. (2001). A role for histone deacetylase HDAC1 in modulating the transcriptional activity of MyoD: inhibition of the myogenic program. Embo J *20*, 1739-1753.
Qiu, Y., Stavreva, D.A., Luo, Y., Indrawan, A., Chang, M.R., and Hager, G.L. (2011). Dynamic Interaction of HDAC1 with a Glucocorticoid Receptor-regulated Gene Is Modulated by the Activity State of the Promoter. J Biol Chem *286*, 7641-7647.
Zhao, P.P., Wang, H.N., Wang, H., Dang, Y.N., Luo, L., Li, S., Shi, Y., Wang, L.F., Wang, S.H., Mager, J., *et al.* (2020). Essential roles of HDAC1 and 2 in lineage development and genome-wide DNA methylation during mouse preimplantation development. Epigenetics-Us *15*.

Original submission second decision letter

MS ID#: DEVELOP/2021/200346

MS TITLE: The histone deacetylase activity of HDAC1/2 is required to safeguard zygotic genome activation in mice and cattle

AUTHORS: Yanna Dang, Shuang Li, Panpan Zhao, Lieying Xiao, Lefeng Wang, Yan Shi, Lei Luo, Shaohua Wang, Huanan Wang, and Kun Zhang

ARTICLE TYPE: Research Article

Dear Dr. 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 from their reports, the referees recognise the potential of your work, but they also raise significant concerns about it. Given the nature of these concerns, I am afraid I have little choice other than to reject the paper at this stage.

While I recognise that the datasets that you have generated would be a huge and important resource for the community, as well as the findings on the potential cross-talk between HDAC1/2 with H3K4me3, we remain concerned about the potential inaccurate message that the manuscript would convey regarding specificity and role of acetylated residues. This is primarily because of the known promiscuous activity of HDAC1/2 towards histones and non-histone targets, which, while valid for H3K27ac, could potentially also affect other targets, which the manuscript does not analyse nor discusses. Because of this, I am sorry that I cannot offer to move ahead with publication of your manuscript, and I regret that these points have not been fixed during revision, but I simply do not have the level of support from our Reviewers that we need, who instead have raised concerns against publication in its current form.

Yours sincerely,

Maria Elena Torres-Padilla
Handling Editor
Development

Reviewer 1

Advance Summary and Potential Significance to Field

This manuscript by Dang and colleagues addresses the function of Hdac1/2 at ZGA in mammalian embryos. They find that over-expression of a catalytically inactive Hdac1 or 2 (H1/H2MU), but not wild-type Hdac leads to a 2-cell stage block, suggesting defects in ZGA. Indeed they find widespread defects in gene expression at the late 2-cell stage, the time of ZGA in mice. They suggest this function is conserved in cattle as inhibition of Hdac1/2 activity results in similar defects in bovine embryo ZGA and development. Previously it has been demonstrated that Hdac1 is important for the regulation of gene expression and preimplantation development in mice. Here the authors go further by suggesting that the catalytic activity of Hdac1 is responsible for this phenotype, through the use of H1MU overexpression. Here I have significant concerns over the experimental design (see below). They go on to suggest that H1MU expression leads to a failure of broad H3K4me3 domain reprogramming at ZGA in mice, and show that the phenotype of H1MU can be partially rescued by expression of a H3K4me3 demethylase, Kdm5b. This finding is particularly interesting and suggests an interplay between histone acetylation and H3K4me3 remodeling during early mammalian development.

Comments for the author

Please find my comments to the responses of the authors below:
1. While the authors have corrected the 2 examples presented, the overall tone of the manuscript still suggests that the effect of Hdac1/2 is via H3K27ac.
There is no analysis of other histone acetylation substrates of Hdacs1/2. In its current form I think the manuscript over-focuses on H3K27ac as the only potential mediator of the Hdac1 effect and thus gives a potentially misleading account of the role of Hdac1/2 in ZGA and embryonic development.
2. The arguments presented by the authors are not sufficient to rule out indirect effects due to overexpression of the mutant enzymes. There is no debate over whether the H141A mutation blocks enzymatic activity or the overexpression of H1MU consistently effects development, as argued by the authors. My concern is more general regarding the experimental design through the overexpression of a mutant enzyme, in the context of an active endogenous enzyme. No experiments are presented to show a true dominant negative action of the mutant enzyme towards the endogenous enzyme on a molecular level. How do the authors rule out an artificial and toxic action of the mutant enzyme in the injected embryos, not related to the endogenous enzyme?
3. In Figure 5b, why is Dux transcript not detectable in wild-type embryos at the early 2-cell stage, as shown by previous literature (e.g. Hendrickson et al., Nat. Genet. 2017, De laco et al., Nat. Genet. 2017)?
4. Addressed.
5. Addressed.
6. Addressed.
7. The requested analysis was not performed.

Therefore, because the most important concerns that give rise to doubt over the conclusions of this study have not been properly addressed, I would not support publication of this manuscript in its current from. Notably I still have significant concerns over the experimental design and the misleading over- emphasis on H3K27ac. Thus the conceptual advance over previous studies in identifying the importance of the catalytic activity of Hdac1 and 2 in ZGA during early mammalian development is not fully supported in my view.

Reviewer 2

Advance Summary and Potential Significance to Field

I greatly appreciated reading this manuscript by Yanna Dang and collaborators investigating the role of histone deacetylase activity of HDAC1/2 in mice and cattle preimplantation development. The results obtained are clear and show that HDAC1/2 is a critical histone modifier that participates in the regulation of ZGA.

Comments for the author

I would like to thank the authors who answered all my comments and modified the manuscript accordingly.
I have no further comments.

Second revision

Author response to reviewers' comments

We are grateful to all referees and editors again for their constructive comments and advices. We are very sorry for not addressing the concerns completely in the previous version. Here, we have revised the manuscript based on suggestions and carried out additional experiments to make our conclusions more convincing in the current version. The point-by-point responses to comments could be found as below. We hope that all these changes fulfill the requirements to make the manuscript acceptable for publication in Development. Please let us know if any further clarifications are necessary.

Point-by-point responses:
Reviewer 1 Advance Summary and Potential Significance to Field:
This manuscript by Dang and colleagues addresses the function of Hdac1/2 at ZGA in mammalian embryos. They find that over-expression of a catalytically inactive Hdac1 or 2 (H1/H2MU), but not wild-type Hdac leads to a 2-cell stage block, suggesting defects in ZGA. Indeed they find widespread defects in gene expression at the late 2- cell stage, the time of ZGA in mice. They suggest this function is conserved in cattle as inhibition of Hdac1/2 activity results in similar defects in bovine embryo ZGA and development. Previously it has been demonstrated that Hdac1 is important for the regulation of gene expression and preimplantation development in mice. Here the authors go further by suggesting that the catalytic activity of Hdac1 is responsible for this phenotype, through the use of H1MU overexpression. Here I have significant concerns over the experimental design (see below). They go on to suggest that H1MU expression leads to a failure of broad H3K4me3 domain reprogramming at ZGA in mice, and show that the phenotype of H1MU can be partially rescued by expression of a H3K4me3 demethylase, Kdm5b. This finding is particularly interesting and suggests an interplay between histone acetylation and H3K4me3 remodeling during early mammalian development.
Response: Thank you for your summary and insightful comments. We have addressed the concern as detailed below.

Reviewer 1 Comments for the Author:
Please find my comments to the responses of the authors below:
While the authors have corrected the 2 examples presented, the overall tone of the manuscript still suggests that the effect of Hdac1/2 is via H3K27ac. There is no analysis of other histone acetylation substrates of Hdacs1/2. In its current form I think the manuscript over-focuses on H3K27ac as the only potential mediator of the Hdac1 effect and thus gives a potentially misleading account of the role of Hdac1/2 in ZGA and embryonic development.
Response: Thank you for the comment. We are so sorry for not addressing this concern clearly enough in the previous version. To tone down the description of H3K27ac, we have re-organized the abstract and first three parts in results of the manuscript. Moreover, we have made substantial revisions for the related information in the introduction (Line 73-85) and discussion section (Line 357-408) to avoid overstating H3K27ac.
Moreover, we analyzed other 4 histone acetylation substrates of Hdac1/2s: H3K14ac, H3K18ac, H4K5ac and H4K16ac. Results show similar changes with H3K27ac in H1MU embryos (Fig. R1A-D). We have added these data and related discussion into the revised manuscript.
Last but not least, we have added a statement to discuss the limitation of the present work (Line 402-408) as “Previous studies have shown HDAC1/2’s substrates include not only histones but also non-histones(Luo et al., 2000; Nalawansha et al., 2017). Here, we found that HDAC1/2’s activity is required for deacetylation at certain lysine sites of histones. In particular, we determined the changes in H3K27ac given its well- established association with active gene

expression. However, we cannot rule out the possibility that the inhibition of HDAC1/2 could potentially affect other substrates, including non-histones, which warrants further investigations in the future."

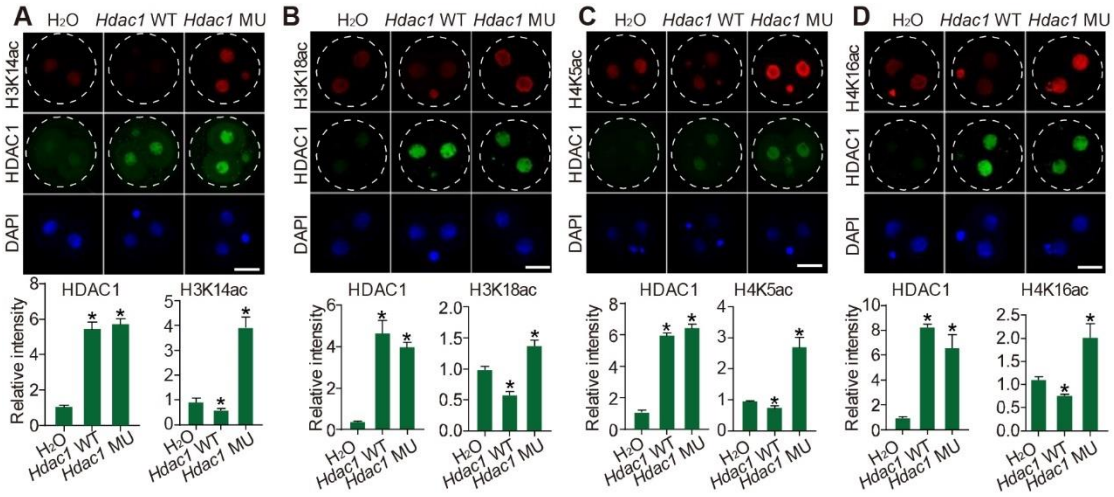

Fig. R1: The histone deacetylase activity of HDAC1 is required for histone deacetylation during ZGA. Top: Immunofluorescence staining of HDAC1 and H3K14ac (A), H3K18ac (B), H4K5ac (C), H4K16ac (D) in late 2-cell embryos. Bottom: HDAC1, H3K14ac, H3K18ac, H4K5ac, and H4K16ac intensity relative to embryos injected with H2O. Data shown as Means ± s.e.m. (n = 3; 3-10 embryos per group per replicate, *P < 0.05 (Hdac1 WT or MU vs H2O)).

2. The arguments presented by the authors are not sufficient to rule out indirect effects due to overexpression of the mutant enzymes. There is no debate over whether the H141A mutation blocks enzymatic activity or the overexpression of H1MU consistently effects development, as argued by the authors. My concern is more general regarding the experimental design through the overexpression of a mutant enzyme, in the context of an active endogenous enzyme. No experiments are presented to show a true dominant negative action of the mutant enzyme towards the endogenous enzyme on a molecular level. How do the authors rule out an artificial and toxic action of the mutant enzyme in the injected embryos, not related to the endogenous enzyme?

Response: Thanks for the comment. To address the concern on artificial and toxic action of the mutant enzyme, we have performed additional analyses or experiments as detailed below.

1. To determine the functional consequence of loss of function of HDAC1/2, FK228, a specific inhibitor of HDAC1/2, was also used in the present study. By clustering analysis of the RNA-seq data, we found the transcriptome of *Hdac1* MU, *Hdac2* MU group is close with FK228 group (Fig. R2A), hence, the changes elicited in *Hdac1* MU or *Hdac2* MU were more likely ascribed to the inhibition of HDAC1 or HDAC2 rather than nonspecific toxic action.

2. If there was artificial or toxic action of *Hdac1* MU, we would see a similar cellular arrest if we overexpressed *Hdac1* MU at a developmental stage. For this question, we microinjecting *Hdac1* MU in combination with *RFP* mRNA (or *RFP* alone as negative control) into one blastomere of late 2-cell embryos in mice (Fig. R2B). Results clearly show *Hdac1* MU-injected blastomeres at 2-cell stage could cleave at least twice in comparison with the phenotype of 2-cell arrest when expressing *Hdac1* MU at the zygote stage (Fig. R2B and R2C).

3. Moreover, we tested the artificial or toxic action of mouse *Hdac1* MU in bovine zygotes (Fig. R2D). The increased HDAC1 and H3K27ac intensity (Fig. R2E) confirmed effective overexpression of mutant HDAC1 and inhibition of deacetylase activity in bovine embryos. Importantly, *Hdac1* MU-injected embryos were arrested at 8/16-cell stage (corresponding to ZGA in cattle, Fig. R2D) rather than 2-cell stage. This result indicates that *Hdac1* MU does not affect embryo cleavage before 8/16-cell stage in cattle, ruling out the possibility of artificial or toxic action of *Hdac1* MU.

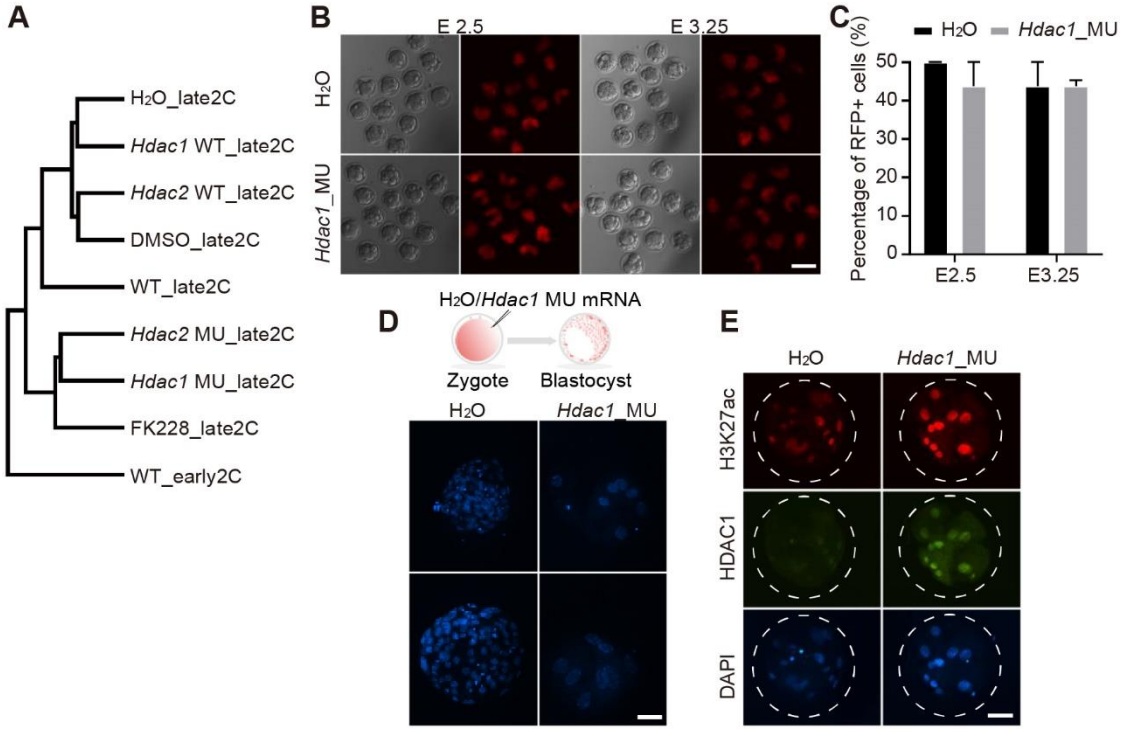

Fig. R2: Evidences for specificity of H1MU. A, Hierarchical clustering of mouse late 2- cell embryos in different groups and wildtype embryos (WT_late2C, WT_early2C(Wu et al., 2016)) based on the FPKM in RNA-seq data. B, Mouse embryos were injected with H2O+*Rfp* and H1MU+*Rfp* mRNA into one blastomere at late 2-cell stage, and the images showed developmental state and RFP fluorescence of embryos on day 2.5 and 3.25 after fertilization. C, Percentage of blastomeres (RFP+) at E2.5 and E3.5 that derived from injected blastomere. D, Experimental scheme for H1MU mRNA injection (top) and DNA staining with DAPI (bottom) at day 8.0 after fertilization for cattle embryos. E, H3K27ac and HDAC1 immunofluorescence staining for bovine 8/16-cell embryos.

3. In Figure 5b, why is Dux transcript not detectable in wild-type embryos at the early 2-cell stage, as shown by previous literature (e.g. Hendrickson et al., Nat. Genet. 2017, De laco et al., Nat. Genet. 2017)?
Response: Sorry about the confusion. We have not performed RNA-seq in that group since we only used H1WT as the control group at early 2-cell stage. We have revised the description and corresponding legends to avoid confusion.

4. Addressed.
5. Addressed.
6. Addressed.
7. The requested analysis was not performed.
The original comment 7 was: The analysis of rescued genes after Kdm5b expression is seriously flawed in my view (Fig. 7e). According to the legend, full rescue refers to genes upregulated (log2 FC ≥ 0) comparing Kdm5b + H1MU vs H1WT. Why is the full rescue effect of Kdm5b in H1MU conditions compared to H1WT conditions? They should compare gene expression levels to wild-type embryos (H20 injected), by selecting differentially expressed genes from Fig 3 – compared to H20 – injected embryos, not H1WT embryos) and determining which percentage of those genes are still differentially expressed or not after H1MU + Kdm5b injection, compared again to H20 injected (using the same thresholding as applied in Fig. 3).”,
Response: We are so sorry for misunderstanding your comment before. As suggested, we performed RNA-seq analysis as described below.
First, we performed analysis of differential expressed genes by comparing H1MU with H2O-injected group (Downregulated genes: Padj<=0.05 and log2FoldChange (H1MU/H2O-injected)<=-1; Upregulated genes: Padj<=0.05 and log2FoldChange (H1MU/H2O-injected)>=1; Fig. R3A). Then, we determined the percentage of these differentially expressed genes in H1MU+Kdm5b

group by comparing with H2O- injected group.
Full rescue genes refers to those downregulated genes that are not differentially expressed in H1MU+Kdm5 group (Padj>0.05 or log2FoldChange (H1MU+Kdm5b/H2O-injected)>-1). Partial rescue genes refers to those downregulated genes that still downregulated in H1MU+Kdm5b but partially rescued compared to H1MU group with log2FoldChange (H1MU+Kdm5b/H1MU+H2O) >0.5. The rest of downregulated genes are “no rescue” genes.
Similarly, Full rescue genes refers to those upregulated genes that are not differentially expressed in H1MU+Kdm5 group (Padj > 0.05 or log2FoldChange (H1MU+Kdm5b/ H2O-injected) < 1). Partial rescue genes refers to those upregulated genes that still upregulated in H1MU+Kdm5b but partially rescued compared to H1MU group with log2FoldChange (H1MU+Kdm5b/H1MU+H2O) < -0.5. The rest of upregulated genes are “no rescue” genes.
As shown in Fig. R3B and 3C, 53.1% and 39.6% of downregulated and upregulated genes could be rescued (Full and partial) by Kdm5b, respectively. We have revised the corresponding figure and legends in the revised manuscript.

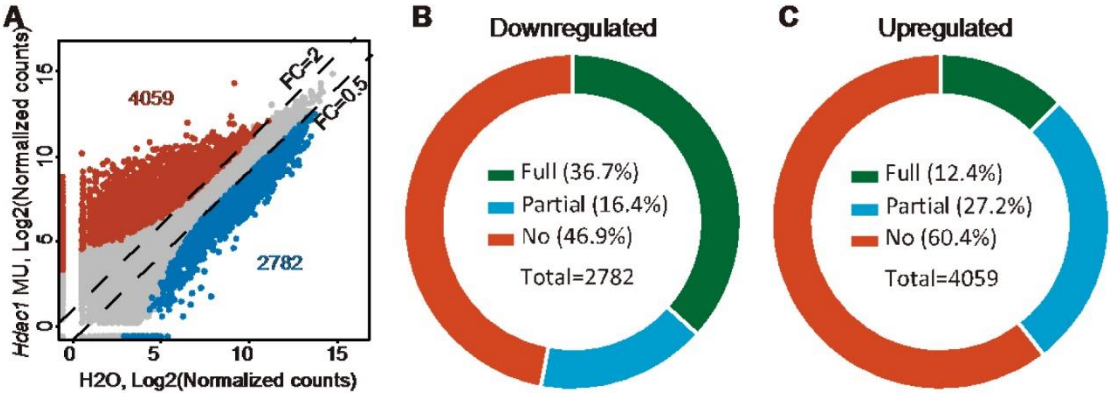

Fig. R3: Rescue effect of *Kdm5b* on the transcriptome. A, Scatter plots showing global gene expression in embryos injected with H1MU mRNA or H2O. The read counts are normalized by DESeq2. Dash lines indicate the threshold of fold change (H1MU/H2O), and grey dots refer to genes with *Padj* > 0.05, while dots in red and blue refer to genes with *Padj* <= 0.05 and fold change (H1MU/H2O) >=2 or <= 0.5. Numbers of up- and down- regulated genes are indicated in the figures. B and C, Donut chart of downregulated genes (H1MU/H2O, B) and upregulated genes (H1MU/H2O, C) based on their extent of rescue in H1MU+Kdm5b mRNA injection embryos.

Therefore, because the most important concerns that give rise to doubt over the conclusions of this study have not been properly addressed, I would not support publication of this manuscript in its current from. Notably I still have significant concerns over the experimental design and the misleading overemphasis on H3K27ac. Thus the conceptual advance over previous studies in identifying the importance of the catalytic activity of Hdac1 and 2 in ZGA during early mammalian development is not fully supported in my view.
Response: Thanks for your suggestions, we hope our responses above could address the concerns over the misleading overemphasis of H3K27ac and artificial effect of H1MU.

Reviewer 2 Advance Summary and Potential Significance to Field:
I greatly appreciated reading this manuscript by Yanna Dang and collaborators investigating the role of histone deacetylase activity of HDAC1/2 in mice and cattle preimplantation development. The results obtained are clear and show that HDAC1/2 is a critical histone modifier that participates in the regulation of ZGA.

Reviewer 2 Comments for the Author:
I would like to thank the authors who answered all my comments and modified the manuscript accordingly. I have no further comments.
Response: Thanks again for your recognition of our work.

Resubmission

First decision letter

MS ID#: DEVELOP/2022/200854

MS TITLE: The lysine deacetylase activity of HDAC1/2 is required to safeguard zygotic genome activation in mice and cattle

AUTHORS: Yanna Dang, Shuang Li, Panpan Zhao, Lieying Xiao, Lefeng Wang, Yan Shi, Lei Luo, Shaohua Wang, Huanan Wang, and Kun Zhang
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.

Reviewer 1

Advance summary and potential significance to field

I thank the authors for addressing the outstanding points and I am now happy to support publication of the manuscript in its current form.

Comments for the author

None