Regulation of paternal 5mC oxidation and H3K9me2 asymmetry by ERK1/2 in mouse zygotes

Baobao Chen, Mingtian Deng, Meng-Hao Pan, Shao-Chen Sun* and Honglin Liu*

Abstract

Background: Extracellular-signal-regulated kinase (ERK) directs cell fate determination during the early development. The intricate interaction between the deposition of H3K9me2, de novo 5mC, and its oxides affects the remodeling of zygotic epigenetic modification. However, the role of fertilization-dependent ERK in the first cell cycle during zygotic reprogramming remains elusive.

Methods: In the present study, we used the small molecule inhibitor to construct the rapid ERK1/2 inactivation system in early zygotes in mice. The pronuclear H3K9me2 deposition assay and the pre-implantation embryonic development ability were assessed to investigate the effect of fertilization-dependent ERK1/2 on zygotic reprogramming and developmental potential. Immunofluorescence and RT-PCR were performed to measure the 5mC or its oxides and H3K9me2 deposition, and the expression of related genes.

Results: We reported that zygotic ERK1/2 inhibition impaired the development competence of pre-implantation embryos. Following the ERK1/2 inhibition, H3K9me2, as well as 5mC and its oxides, were all accumulated abnormally, and the excess accumulation of paternal H3K9me2 and 5mC resulted in reduced asymmetry between parental pronuclei. Furthermore, ERK1/2 inhibition triggered paternal pronuclear localization of the H3K9 methyltransferase G9a and Tet methylcytosine dioxygenase 3 (Tet3). Moreover, the excess localization of G9a antagonized the tight binding of Tet3 to paternal chromatin when ERK1/2 was inhibited.

Conclusions: In conclusion, we propose that zygotic H3K9me2 and 5mC are regulated by fertilization-dependent ERK1/2, which contributes to the development competence of pre-implantation embryos in mice.

Keywords: ERK1/2, H3K9me2, 5mC, Paternal pronucleus, Zygotic reprogramming

Background

The paternal and maternal pronuclei show asymmetric epigenetic marks [1], chromatin structure [2, 3], and transcriptional activity [4, 5] in the zygotic reprogramming. For example, the paternal pronucleus exhibits higher transcriptional activity [4, 5], and less condensed chromatin than the maternal pronucleus in mice [2, 6]. During the zygotic reprogramming, the paternal genome initiates widespread deposition of de novo epigenetic marks, making it an excellent model to understand how epigenetic marks are loaded on the genome orderly [1, 2, 7].

In mammals, mitogen-activated protein kinases (MAPKs) regulate the stability of the maternal and zygotic transcripts [8] and are essential for the early development of mouse zygotes [9]. It was reported that the MII-phase oocytes are stabilized by appropriate levels of MPF [10] and MAPKs [11]. After sperm-oocyte fusion, the decrease of MPF activity (within 10 min)
The zygotic genome exhibits an asymmetric H3K9me2 pattern. The maternal pronuclei remain considerable H3K9me2, while paternal pronuclei bear no H3K9me2 [19–21]. Subsequently, the paternal genome progressively gains H3K9me2 from the late zygote to the cleavage stage [19, 21], suggesting fine regulation of H3K9me2 during zygotic reprogramming. G9a (also known as EHMT2 and KMT1C) is responsible for this progressive deposition of paternal H3K9me2 [22], and it protects the maternal pronucleus from 5mC oxidation [22]. During embryogenesis, G9a controls transcription levels of zygotic genes [23]. These studies suggest that G9a plays pivotal roles during zygotic reprogramming [21, 22].

5mC is one of the well-documented epigenetic factors associated with gene silencing and plays an important role in facilitating the propagation of cellular identity through cell divisions [24, 25]. Meanwhile, 5-hydroxymethylcytosine (5hmC), the oxide of 5mC [26], is positively correlated with gene expression and plays an important role in epigenetic regulation and genome reprogramming during mammalian development [1, 7, 25, 27]. It was reported that the sperm-derived genome undergoes actively demethylated through Tet3-mediated 5mC oxidation, whereas the maternal genome is passive dilution by DNA replication [28, 29]. Importantly, Tet3 also contributes to paternal demethylation by countering the de novo 5mC [30]. While immunostaining results support a role for H3K9me2 in protecting 5mC [31, 32], many bisulfite sequencing (BS-seq) results show that 5mC in mammals is largely independent of H3K9me2 [33, 34], suggesting unknown factors regulated the cross-talk between H3K9me2 and 5mC.

Therefore, in the present study, we examined the role of zygotic ERK1/2 during preimplantation development and reported the fine regulation of paternal 5mC oxidation and H3K9me2 asymmetry by ERK1/2, which provide insights into the role of ERK1/2 during zygotic reprogramming.

**Results**

**Zygotic ERK1/2 is essential for preimplantation development**

We first analyzed the expression of MAPKs during early embryo development by re-analysis the RNA-seq data-sets of mouse embryos. As shown in Fig. 1A, B, MAPKs, specifically ERK1/2, were highly expressed during zygotic genome activation (ZGA), suggesting that MAPKs might play pivotal roles during ZGA in mice. To confirm the hypothesis, U0126, GDC-0994, SB203580, and SP600125 were used to inhibit the protein of MEK1/2, ERK1/2, p38, and JNK, respectively. We selected the maximum concentration before the morphology of zygotes becomes abnormal as the working concentration for short-term treatment (Additional file 1: Fig. S1), and the suppression effect of these inhibitors at the working concentration has been verified (Additional file 1: Fig. S2). As H3K9me2 deposition is critical for preimplantation [34], we analyzed the H3K9me2 signal in zygotes. As shown in Fig. 1C, the signal intensity of H3K9me2 was increased in ERK1/2-inhibited paternal and maternal pronuclei compared to the controls (p < 0.01). Similar results were found in both MEK1/2 and p38-inhibited paternal and maternal pronuclei (p < 0.01). Meanwhile, the paternal/ maternal signal ratio of H3K9me2 was greatly increased in ERK1/2-inhibited zygotes (p < 0.01), but decreased in both MEK1/2 and p38-inhibited zygotes (p < 0.01; Fig. 1C, D). Of note, H3K9me2 signal intensity was also increased in ERK1/2-inhibited paternal pronuclei when compared to that of MEK1/2 and p38-inhibited paternal pronuclei (p < 0.01).

The zygotes were further cultured 85 h after inhibition of ERK1/2 (Fig. 1E). As expected, the ERK1/2-inhibited embryos showed a decreased percentage of blastocysts in both 50 μM (60.48 ± 1.91% vs. 76.42 ± 1.39%, p < 0.01) and 100 μM GDC-0994 treatment group (39.05 ± 2.9% vs. 76.42 ± 1.39%, p < 0.01; Fig. 1F, G). Specifically, 100 μM GDC-0994 treatment led to an abnormality at the 2-, and 4-cell with a percentage of 16.61 ± 1.39% (p < 0.05) and 29.8 ± 0.936% (p < 0.01), respectively. Considering the effects of Oct4 and Nanog on lineage differentiation in the early embryo [35–39], we further investigated the transcription of several pluripotent genes in morula and blastocyst (Additional file 1: Fig. S3 and Fig. 1H). The transcription of Oct4 (p < 0.05), Nanog (p < 0.05), Sox2 (p < 0.05), and Klf4 (p < 0.05) were all highly expressed in ERK1/2-inhibited blastocysts (Fig. 1H). Taken together, these data suggest that zygotic ERK1/2 is essential for embryo preimplantation development in mice.
**Fig. 1** The zygotic ERK1/2 is essential for preimplantation development. A, B Heatmap and boxplot revealed dynamic expression of MAPKs during early embryo development. C H3K9me2 deposition in zygotes treated with inhibitors of different MAPKs pathways at PN4–5 (10 hpf). DNA is stained using Hoechst 33342 (blue). Scale bars, 20 µm. D Quantification is represented as the mean of H3K9me2 signal intensity in pronuclei after background subtraction (left axis) or a ratio between parental pronuclei signals (pat./mat., right axis). Each data point represents an independent zygote. Number of zygotes analysed for each group: control n = 22; iMEK1/2 + ip38 n = 27; iERK1/2 (100 μM GDC-0994) n = 19. E Time scheme of zygote collection and embryo recovery. F Representative bright-field images of blastocysts recovered from the control (left), the 50 μM (middle), and 100 μM (right) GDC-0994-treated group. Black asterisks indicate examples with normal morphology; black arrows denote the abnormal embryos with the reduced cavity. Scale bars, 100 µm. G Percentage of abnormal embryos after ERK1/2 inhibition. From three independent experiments (total number of embryos analysed: 2-cell embryos, n = 205 for Control, n = 193 for 50 μM GDC-0994 treatment, n = 209 for 100 μM GDC-0994 treatment; 4-cell embryos, n = 198 for Control, n = 176 for 50 μM GDC-0994 treatment, n = 176 for 100 μM GDC-0994 treatment; blastocysts, n = 172 for Control, n = 134 for 50 μM GDC-0994 treatment, n = 97 for 100 μM GDC-0994 treatment). H Upregulation of Oct4, Nanog, Sox2, and Klf4 in GDC-0994 treatment blastocysts as revealed by quantitative PCR. P values are indicated. Error bars indicate SD. ♂, maternal pronucleus; ♀, paternal pronucleus. PB, polar body.
ERK1/2 regulates the H3K9me2 and DNA methylation reprogramming in paternal genomes

We next investigated whether the dynamic change of H3K9me2 is ERK1/2 dose-dependent. As shown in Fig. 2A, B, the signal intensity of H3K9me2 was not statistically changed in 50 μM GDC-0994 treatment zygotes compared to the controls. However, in 100 μM GDC-0994 treatment zygotes, the level of H3K9me2 signal was markedly increased in both the paternal and maternal pronuclei (p<0.01), and the paternal to

Fig. 2  ERK1/2 regulates the H3K9me2 and DNA methylation reprogramming in paternal genomes. A H3K9me2 staining of control, 50 μM GDC-0994-treated, and 100 μM GDC-0994-treated zygotes at PN4-5 stage (10 hpf). B Values are represented as the mean of H3K9me2 signal intensity in paternal and maternal pronuclei after background subtraction (left axis) or as a ratio between signals of parental pronuclei (pat./mat., right axis). Number of zygotes analysed for each group: control n=10; 50 μM GDC-0994-treated n=15; 100 μM GDC-0994-treated n=15. C 5mC (green) and 5caC (red) staining of control, 50 μM GDC-0994-treated, and 100 μM GDC-0994-treated zygotes at PN4-5 stage (10 hpf). D, E Quantification of 5mC and 5caC is represented as signal intensity in paternal and maternal pronuclei (left axis) or as a ratio between parental signals (pat./mat., right axis). Number of zygotes analysed for each group: control n=23; 50 μM GDC-0994-treated n=34; 100 μM GDC-0994-treated n=28. Statistical analysis was carried out using Student’s t-test (two-sided). P values are indicated. Error bars indicate SD. ♀, maternal pronucleus; ♂, paternal pronucleus. Scale bar, 20 μm
maternal signal ratio of H3K9me2 was also significantly increased (p < 0.01), suggesting that regulation of paternal H3K9me2 is ERK1/2 dose-dependent. We further examined the level of 5mC and 5caC signals in ERK1/2-inhibited zygotes. As shown in Fig. 2C, D, the level of paternal 5mC was increased following treatment with 50 μM GDC-0994 (p < 0.01). The product of the Tet3 oxidation chain, 5caC, was also accumulated more than the controls (p < 0.01) (Fig. 2C, E). Compared with the 50 μM GDC-0994 treated zygotes, the paternal 5mC remained stable in 100 μM GDC-0994-treated zygotes, but the accumulation of paternal 5caC was significantly reduced (p < 0.01), indicating that ERK1/2 might play pivotal roles in the oxidation of paternal 5mC. Notably, the asymmetries of 5mC, 5caC, and H3K9me2 between the paternal and maternal genomes were all decreased following 100 μM GDC-0994 treatment (p < 0.01, p < 0.05, p < 0.01, respectively) (Fig. 2A–E), suggesting that ERK1/2 is involved in the asymmetric regulation of these parental epigenetic marks in zygotes.

**ERK1/2 contributes H3K9me2 asymmetry by preventing paternal G9a localization**

We reported the absence of paternal H3K9me2 in control zygotes in our previous studies [19, 20, 40]. However, paternal H3K9me2 showed pronuclear deposition with progressive enrichment since the early PN3 stage in ERK1/2-inhibited zygotes (Fig. 3A). The quantitative assessment revealed a significant and continuous deposition of paternal H3K9me2 (p < 0.01), while the maternal H3K9me2 increased rapidly and limitedly in ERK1/2-inhibited zygotes (p < 0.01) (Fig. 3B, C). The paternal to maternal signal ratio of H3K9me2 was greatly increased at the early PN3 (p < 0.05), late PN3 (p < 0.01), and PN5 (p < 0.01) in ERK1/2-inhibited zygotes compared to that of the controls (Fig. 3D), suggesting aberrant H3K9me2 deposition in minor ZGA when ERK1/2 was inhibited.

We further investigated the distribution of parental H3K9me2 at 17 hpf and 24 hpf. As shown in Fig. 3E, H3K9me2 was asymmetrically distributed at the 17 hpf and the 24 hpf in the controls. However, the asymmetry of parental H3K9me2 disappeared following treatment with GDC-0994 at the 17 and 24 hpf (Fig. 3E). H3K9me2 is important for protecting the methylation of paternally imprinted gene H19 against active DNA demethylation [31]. The aberrant H3K9me2 asymmetry might impair the expression of H19. As expected, H19 was significantly down-regulated whereas Igf2 was highly expressed in blastocysts of the ERK1/2-inhibited group (Fig. 3F, p < 0.01, p < 0.01, respectively). Our data suggest that zygotic ERK1/2 is responsible for the asymmetry of parental H3K9me2 during ZGA by impeding the deposition of paternal H3K9me2.

Our previous study revealed that G9a is required for paternal H3K9me2 deposition in zygotes [21]. As shown in Fig. 4A, G9a was enriched in both paternal and maternal pronuclei (p < 0.01), and paternal to maternal signal ratio of G9a was increased following 100 μM (p < 0.05), but not 50 μM GDC-0994 treatment compared to the controls (Fig. 4A, B). To confirm that ERK1/2 regulates H3K9me2 asymmetry through G9a, we carried out IVF in the presence of BIX-01294, an inhibitor that competed for the substrate of G9a [41, 42]. Inhibition of G9a showed no statistical change of H3K9me2 in paternal pronuclei. However, the deposition of paternal H3K9me2 induced by ERK inhibition was barely detected in both ERK1/2 and G9a-inhibited zygotes, similar to the controls (Fig. 4C, D). In addition, the paternal pronuclear localization of G9a was not only increased in ERK1/2-inhibited zygotes, but also increased in both ERK1/2 and G9a-inhibited (GDC-0994 + BIX-01294-treated) zygotes (p < 0.01 Fig. 4E, F). These data demonstrate that ERK1/2 impedes paternal localization of G9a and contributes to the formation of H3K9me2 asymmetry between parental pronuclei.

**Inhibition of ERK1/2 promotes the oxidation of de novo 5mC in paternal pronuclei**

Since 5mC and 5caC were both accumulated in parental pronuclei with 50 μM GDC-0994 treatment, we next assessed the dynamic changes of 5hmC in parental pronuclei following 50 μM GDC-0994 treatment. As shown in Fig. 5A–C, both 5mC and 5hmC were increased at the late PN3 in maternal pronuclei with 50 μM GDC-0994 treatment (p < 0.01). In paternal pronuclei, the 5mC signal intensity was increased at the early PN3 (p < 0.05) and late PN3 (p < 0.01), meanwhile, 5hmC level was higher in early PN3, late PN3, and PN5 stage in ERK1/2-inhibited zygotes (p < 0.01) (Fig. 5D, E), suggesting that inhibition of ERK1/2 promotes oxidation of paternal 5mC. Moreover, the paternal to maternal signal ratios of 5mC (p < 0.05) and 5hmC (p < 0.01) were increased at the PN5 stage and the early PN3, respectively (Fig. 5F, G).

We further investigated the roles of DNMT and Tet3 in the yields of paternal 5mC and 5hmC when ERK1/2 was inhibited. Decitabine and DMOG were used for the inhibition of DNMT and Tet3 in zygotes as described previously [30], and the suppression effect has been verified (Additional file 1: Fig. S4 and Fig. 7A, B). As shown in Fig. 6A, B, paternal 5mC level was reduced in both ERK1/2 and DNMT-inhibited zygotes, as well as in DNMT-inhibited zygotes compared with that of ERK1/2 inhibition (p < 0.01). Interestingly, the localization of Tet3 in paternal pronuclei was increased when ERK1/2 was inhibited (p < 0.01) (Fig. 6C, D). However, the 5mC level was not affected by Tet3 inhibition (Fig. 6E, F). These
ERK1/2 impedes the deposition of paternal H3K9me2. A H3K9me2 deposition in ERK1/2-inhibited (100 μM GDC-0994) zygotes at different developmental stages. The pronuclear (PN) stages are indicated. Quantification of the H3K9me2 signal intensity in parental pronuclei (B, C) and the paternal to maternal signal ratio of H3K9me2 (D) in ERK1/2-inhibited zygotes. Number of zygotes analysed for each stage: Early PN3, n = 10; Late PN3, n = 11; PN5, n = 14 for control and Early PN3, n = 9; Late PN3, n = 19; PN5, n = 14 for GDC-0994-treatment. Each data point represents an independent zygote. E Representative images show equal distribution of H3K9me2 between paternal and maternal genomes in ERK1/2-inhibited (100 μM GDC-0994) 2-cell embryos compared to the controls. F The expression of H19 and Igf2 in ERK1/2-inhibited blastocysts. P values are indicated. Error bars indicate SD. ♀, maternal pronucleus; ♂, paternal pronucleus. PB, polar body. Scale bar, 20 μm
data indicate that inhibition of ERK1/2 promotes the accumulation of de novo 5mC in paternal pronuclei. In the absence of de novo 5mC in paternal pronuclei, ERK1/2 inhibition induced 5hmC was eliminated in both ERK1/2 and DNMT-inhibited zygotes (p < 0.01) (Fig. 6A, B), suggesting that DNMT-driven de novo 5mC is required for the excess accumulation of paternal 5hmC in ERK1/2-inhibited zygotes. We
further investigated whether the excess paternal 5hmC in ERK1/2-inhibited zygotes was produced by Tet3. As shown in Fig. 6E, the excess accumulation of paternal 5hmC induced by ERK1/2 inhibition disappeared in both ERK1/2 and Tet3-inhibited zygotes (p < 0.01).

Taken together, our results demonstrate that ERK1/2 inhibition promotes Tet3-driven oxidation of excess de novo 5mC in paternal pronuclei, and subsequently leads to accumulation of paternal 5hmC.
Fig. 6 (See legend on previous page.)
Excess localization of G9a impedes oxidation of 5mC in paternal pronuclei
Since the pronuclear localization of G9a and Tet3 was both increased after ERK1/2 inhibition, and the GDC-0994 concentration required for accumulation of paternal Tet3 was lower than that of paternal G9a, we further assessed the interaction between G9a and Tet3. Under the TP conditions, we found reduced tight binding of Tet3 to chromatin following DMOG treatment, especially in paternal pronuclei (p < 0.01) (Fig. 7A, B). Conversely, the G9a localization was increased significantly in paternal pronuclei (p < 0.01), but not in maternal pronuclei with DMOG treatment (Fig. 7C, D). Moreover, paternal Tet3 was decreased in G9a-inhibited (BIX-01294-treated) zygotes (p < 0.01) (Fig. 7E, F). These data suggest that the accumulation of pronuclear G9a resulted in the reduction of Tet3 localization in paternal pronuclei.

To validate the interaction between Tet3 and G9a, we detected the level of paternal 5mC and 5hmC signals in the presence of both ERK1/2 and Tet3 inhibitors. As shown in Fig. 7G, H, both inhibition of ERK1/2 and G9a catalytic activity (50 μM GDC-0994 and BIX-01294, respectively) resulted in the accumulation of paternal 5mC (p < 0.05) and the loss of paternal 5hmC (p < 0.01) compared to the ERK1/2-inhibited zygotes. Since inhibition of both ERK1/2 and G9a led to a loss of H3K9me2 in paternal pronuclei, these data suggest that excess localization of G9a impedes oxidation of 5mC in paternal pronuclei by inhibition of Tet3. Moreover, paternal H3K9me2 was increased in both ERK1/2 and Tet3-inhibited (50 μM GDC-0994 and DMOG, respectively) zygotes (Fig. 7I, J), suggesting that Tet3 impedes the paternal H3K9me2 deposition in zygotes as well.

Discussion
In the present study, we found that fertilization-dependent ERK1/2 is the critical regulator for early embryo development in mice. We further reported reduced asymmetries of 5mC, 5hmC, 5caC, and H3K9me2 at the PN stage after ERK1/2 inhibition. In addition, ERK1/2 inhibition led to excess pronuclear localization of G9a, which subsequently antagonizes the tight binding of Tet3 to paternal chromatin, providing crucial insights into the regulation of DNA methylation and histone modifications crosstalk by ERK1/2.

Previous studies revealed that ERK1/2 protein levels increase after fertilization [14, 43, 44], which is the minor ZGA. Consistently, we found that ERK1/2 was highly expressed during ZGA, as revealed by RNA sequencing data. We further observed that multiple MAPKs pathways are involved in the regulation of the maternal H3K9me2 deposition. As for paternal genomes, H3K9me2 deposition showed the most significant increase, and the asymmetry of the parental H3K9me2 was altered following zygotic ERK1/2 inhibition, indicating that ERK1/2 plays an important role in the regulation of paternal H3K9me2 and the asymmetry between the parental genomes.

It was demonstrated that mouse minor ZGA occurs at the PN3 stage, during which, the first zygotic transcription is promiscuous [45] and the epigenetic modifications are rapidly reprogrammed [5, 30, 31, 46]. In the present study, we found decreased levels of 5mC and H3K9me2 in maternal pronuclei from the PN3 stage, consistent with previous studies [21, 47, 48]. Considering that 5mC and H3K9me2 are transcriptional repressive markers [24, 25, 49, 50], the removal of 5mC and H3K9me2 would be important for the initiation of minor ZGA. Indeed, studies reported the symmetry distribution of 5mC between paternal and maternal pronuclei at PN stage impairs the embryonic development [32] and that of H3K9me2 is embryonic lethal [22], suggesting the asymmetries distribution of 5mC and H3K9me2 are critical for the early embryo development.

A recent study revealed that ERK is related to transcription activity and the fluctuation or persistence of ERK signal would irreversibly change the expression of pluripotency genes [51]. In the present study, the expression of two genes Oct4 and Nanog, which serve as hubs in the core pluripotency network, was increased in the expanded and hatched blastocysts after zygotic ERK1/2 inhibition, suggesting that the regulation of ERK on the
Fig. 7 (See legend on previous page.)
determination of the embryonic fate occurs in the first cell cycle, consistent with the results of directing fate specification in the preimplantation embryo by ERK [15]. The level of the 5hmC signal was increased in both 
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paternal and maternal pronuclei at the PN3 stage following 
treatment with a low concentration of ERK1/2 inhibitor. A previous study reported the suppression of 
Tet1 by the ERK pathway in cells [52]. However, Tet1 was 
barely expressed at the PN stage. Therefore, we focused 
on Tet3, which has previously been reported as a key 
regulator of the 5mC asymmetry between paternal and 
maternal pronuclei [30, 31, 53]. As expected, the Tet3 
localization in paternal pronuclei was increased following 
the treatment with a low concentration of ERK1/2 inhibi-
tor, suggesting that ERK1/2 controls the process of pater-
nal 5mC oxidation by preventing pronuclear localization 
of Tet3 in the zygotic genome.

G9a is essential for preimplantation development [34, 
54] and limits the range of the promiscuous transcrip-
tions during exposure to stress [55]. Following the treat-
ment with a higher concentration of ERK1/2 inhibitor, the 
H3K9me2 deposition and G9a localization in paternal 
pronuclei were both increased. Meanwhile, the num-
ber of blastocysts from zygotes with the same treatment 
was decreased sharply, suggesting that the limitation 
of G9a on the promiscuous transcription of the zygotic 
genome and the lethality of excess H3K9me2 deposition 
in paternal pronuclei during early development.

Our study shows that G9a plays a vital role in 5mC 
oxidation during paternal genomic reprogramming. The 
excess pronuclear localization of G9a antagonized the 
tight binding of Tet3 to paternal chromatin and effec-
tively blocked the accumulation of paternal 5hmC in both 
ERK1/2 and G9a-inhibited zygotes, suggesting that ferti-
lization-dependent ERK1/2 promotes rapid oxidation of 
paternal 5mC by inhibiting G9a pronuclear localization.

The catalytic activity of G9a has previously been reported 
that essential for protecting the maternal genomic meth-
hylation from Tet3-mediated 5mC oxidation [22]. Interest-
ingly, in both ERK1/2 and Tet3-inhibited zygotes where 
G9a pronuclear localization increased and catalytic activ-
ity was maintained, the paternal genomes showed a sig-
ificant H3K9me2 deposition and a downward trend in 
de novo 5mC accumulation that ERK1/2 inhibition pro-
duced. Furthermore, compared with paternal genomes, 
the accumulation of de novo 5mC and 5hmC in mater-
nal genomes both showed a lag after ERK1/2 inhibition. 
These data are consistent with the notion that H3K9me2 
protects the zygotic genome against the de novo 5mC 
[56, 57]. Therefore, our findings support the notion that 
G9a pronuclear localization impedes the oxidation of 
paternal 5mC during zygotic epigenetic reprogramming,
regardless of whether G9a retains its catalytic perfor-
ance or not.

Our study also reveals the crosstalk between 5mC and 
H3K9me2 in the zygotic genome. In our ERK1/2 inhibi-
tion system, Tet3 converts de novo 5mC into 5hmC, 
while the deposition of H3K9me2 prevents the accumula-
tion of de novo 5mC in the zygotic genome. Recently, the 
particular epigenetic landscapes show that 5mC is largely 
independent of H3K9me2 in differentiated [33] and dis-
edeed cells [58, 59]. H3K9me2 protects 5mC inherited 
from gametes, but it might prevent the accumulation of 
de novo 5mC at some loci in zygotes. Therefore, this 
indifference between genomic 5mC and H3K9me2 can 
be explained by DNA replication and the inheritance 
5mC dilution.

Conclusions

We have demonstrated that the inhibition of zygotic 
ERK1/2 causes excess deposition of H3K9me2 and accu-
mulation of 5mC and its oxides in paternal pronuclei by 
triggering the pronuclear localization of G9a and Tet3, 
respectively. In conclusion, we propose that zygotic 
reprogramming is regulated by fertilization-dependent 
ERK1/2, which contributes to the development competen-
tence of pre-implantation embryos in mice.

Materials and methods

Sperm collection

Sperm was obtained from ICR males aged 10–20 weeks. 
The cauda epididymis was cut open with the tip of 
syringes to allow sperm swimming out. A mass of sperm 
was put into Human Tubal Fluid (HTF) fertilization 
medium supplemented with 4 mg/ml bovine serum albu-
min (BSA, Sigma-Aldrich) and incubated for 1–1.5 h at 
37 °C in 5% CO2.

In vitro fertilization

ICR females aged 4–6 weeks were superovulated by 
intraperitoneal injection of 10 U pregnant mare’s serum 
(PMS) and 10 U of human chorionic gonadotropin 
(HCG) 48 h later. Cumulus oocyte complexes collected 
14 h post HCG injection were incubated with capacitated 
sperm for 2.5 h. HCG inhibition was performed by sup-
plementing with 1 mM dimethylallyl glycine (DMOG, 
Sigma-Aldrich) in fertilization medium; oocytes were 
icubated with DMOG for at least 40 min before addi-
tion of sperm.

Zygotes culture and collection

Zygotes were washed off the excess sperm and granulosa 
cells at 2.5 hpf, and were randomly divided into several 
equal parts for different treatments: GDC-0994 (50– 
100 μM, Selleck, #s7554), U0126-EtOH (40 μM, Selleck,
Vectashield and imaged as described above. DNA was stained by 1 μg/ml Hoechst33342 for 15 min, and zygotes were mounted in Vectashield (Vector laboratories) and were visualized with a ×40 objective.

Immunofluorescence staining of 5mC, 5hmC, and 5caC

Zygotes were fixed in 3.7% paraformaldehyde (PFA) for 60 min, and permeabilized in PBS, 1% BSA, 0.5% Triton X-100 for 25 min at room temperature (RT). Zygotes were blocked for 1 h in PBS, 1% BSA at RT and incubated with the following primary antibody: H3K9me2 (Cell Signaling Technology, #4658, 1:100), G9a (Cell Signaling Technology, #3306, 1:100), and Tet3 (Abcam, #ab153724, 1:200) overnight at 4 °C. Zygotes were then incubated with Alexa Fluor 488- or 555-conjugated IgG secondary antibody (Molecular Probes, 1:200) overnight at 4 °C. Zygotes were then incubated with Alexa Fluor 488- or 555-conjugated IgG secondary antibody (Molecular Probes, 1:200) overnight at 4 °C. DNA was stained by 1 μg/ml Hoechst33342 (Beyotime, Shanghai, China) for 15 min, and zygotes were mounted in Vectashield (Vector laboratories) and were visualized using an LSM710 confocal laser scanning microscope (Carl Zeiss) with a 40 objective.

TP conditions are used for Tet3 staining in zygotes. Triton pre-extraction was performed as previously described with minor modifications [30, 31]. Briefly, zygotes were incubated in ice-cold permeabilization solution (50 mM NaCl, 3 mM MgCl₂, 0.5% Triton X-100, 300 mM sucrose in 25 mM HEPES pH 7.4) for 45–60 s until the perivitelline space was eliminated, followed by PFA fixation.

Western blotting analysis

MEK1/2 (Cell Signaling Technology, #8727, 1:1000), phospho-MEK1/2 (Cell Signaling Technology, #9154, 1:1000), ERK1/2 (Cell Signaling Technology, #4695, 1:1000), phospho-ERK1/2 (Cell Signaling Technology, #4370, 1:1000), p38 (Cell Signaling Technology, #8690, 1:1000), phospho-p38 (Cell Signaling Technology, #4511, 1:1000), SAPK/JNK (Cell Signaling Technology, #9252, 1:1000), phospho-SAPK/JNK (Cell Signaling Technology, #4668, 1:1000), Dnmt1 (Abcam, ab188453, 1:1000), Dnmt3a (Abcam, ab188470, 1:2000), and beta Actin (Bioss, bs-0061R, 1:1000) protein expression in zygotes were verified by western blot analysis. 48–150 mouse zygotes per group were placed in ice-cold RIPA lysis buffer (Beyotime, Shanghai, China) containing 1% PMSF (Beyotime, Shanghai, China). The protein was added with 5× SDS-PAGE Sample Loading Buffer (Beyotime, Shanghai, China) and then denatured on a PCR instrument at 100 °C for 10 min. Proteins were separated by SDS-PAGE (GenScript) at 130 V for 60 min and then electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Merck, Millipore, Ltd). After transfer, membranes were blocked with TBST that contained 5% BSA for 2 h, followed by incubation at 4 °C overnight with a rabbit primary antibody. After washing three times in TBST (5 min each), membranes were incubated with Horseradish peroxidase-labeled anti-rabbit IgG secondary antibody (dilution 1:5000; Cell Signaling Technology) for 2 h at RT. Finally, the specific proteins were visualized using Western blotting detection kit (Advansta) and analyzed by ImageJ software. After detection of phosphorylated antigen, the membrane was incubated with membrane regeneration solution (Solarbio) for 60 min for stripping and reprobing of the immunoblot of the total protein levels in the same sample.

Gene expression analysis

RNA-seq data of mice (GSE98150) were downloaded from Gene Expression Omnibus. Gene expression of MAPKs during early embryo development was normalized with log2(count + 1).

Blastocysts (30 for each treatment) were collected at day E4, and mRNA was purified using Dynabeads mRNA DIRECT™ KIT (invitrogen) following the manufacturer’s instructions. Random primed reverse transcription was performed using HiScript III RT SuperMix with gDNA wiper (R323-01, Vazyme, Nanjing, China). cDNA was added to 10 μl of quantitative PCR mix (Q111-02, Vazyme, Nanjing, China). RT-PCR reactions were performed on a Step-One Plus Real-Time PCR system (Applied Biosystems, Carlsbad, CA,
USA). The primers for quantitative analysis are shown in Additional file 2: Table S1. Gene expression was calculated using the 2^ΔΔCt method, and GAPDH was used for normalization as endogenous reference genes.

Data analysis
Images were analyzed using ImageJ software. The midsection of each pronucleus was identified using Hoechst33342 staining and determined by the maximal area. The midsection was used to quantify the total intensity following the subtraction of the signal corresponding to the cytoplasmic area (representing staining background). Statistical analysis was carried out using two-tailed Student's t-test with Welch's correction when required, using GraphPad Prism software. For gene expression analysis, statistical analysis was performed using two-tailed unpaired t-test. At least three biological replicates were performed for each analysis. Each replicate was conducted by an independent experiment at different times.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s13578-022-00758-x.

Additional file 1: Figure S1. Phenotype of zygotes following treatment with inhibitors of MEK1/2, ERK1/2, p38, and JNK. Figure S2. The expression of phosphorylated and total MAPKs proteins in zygotes treated with corresponding inhibitors. Figure S3. The expression of pluripotent genes in morula that zygotic ERK1/2 inhibited. Figure S4. The expression of Dnmt1 and Dnmt3a in DNMT-inhibited zygotes.

Additional file 2: Table S1. Primers used for qRT-PCR analysis.

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Authors' contributions
BC, SCS, and HL conceived and designed the experiments; MD and MHP contributed reagents and materials; BC performed the experiments, analyzed the data, and wrote the paper. BC and SCS discussed the results. BC, SCS, and MD revised the paper. All authors approved the submission of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations
Ethics approval and consent to participate
All animal experiments were followed the rules and guidelines for the Animal Care and Use Committee of Nanjing Agricultural University.

Consent for publication
All co-authors agreed to publish the final version of the present manuscript.

Competing interests
The authors declare that they have no competing interests.

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References
1. Xia W, Xie W. Rebooting the epigenomes during mammalian early embryogenesis. Stem Cell Rep. 2020;15(6):1158–75.
2. Ooga M, Fuka H, Hashimoto S, Suzuki MG, Aoki F. Analysis of chromatin structure in mouse preimplantation embryos by fluorescent recovery after photobleaching. Epigenetics. 2016;11(1):85–94.
3. Flyamer IM, Gassler J, Imakaev V, Brandão HB, Ulíanov SV, Abdenur N, et al. Single-nucleus Hi-C reveals unique chromatin reorganization at oocyte-to-zygote transition. Nature. 2017;544(7648):110–4.
4. Aoki F, Worrad DW, Schultz RM. Regulation of transcriptional activity during the first and second cell cycles in the preimplantation mouse embryo. Dev Biol. 1997;181(2):296–307.
5. Funaya S, Aoki F. Regulation of zygotic gene activation by chromatin structure and epigenetic factors. J Reprod Dev. 2017;63(4):359–63.
6. Bogolyubova I, Bogolyubov D. Heterochromatin morphodynamics in late oogenesis and early embryogenesis of mammals. Cells. 2020;9(6):1497.
7. Marcho C, Cui W, Nager J. Epigenetic dynamics during preimplantation development. Reproduction. 2015;150(3):R109-120.
8. Gonsalves FC, Weisblat DA. MAPK regulation of maternal and zygotic Notch transcript stability in early development. Proc Natl Acad Sci USA. 2007;104(2):531–6.
9. Xu J, Liu T, Han F, Zong Z, Wang G, Yu B, et al. AURKB and MAPK involvement in the regulation of the early stages of mouse zygote development. Sci China Life Sci. 2012;55(1):47–56.
10. Sagata N, Daar I, Oskarsson M, Showalter SD, Vande Woude GF. The product of the mos proto-oncogene as a candidate "initiator" for oocyte maturation. Science. 1989;245(4918):643–6.
11. Chau AS, Shibuya EK. Mos-induced p42 mitogen-activated protein kinase activation stabilizes M-phase in Xenopus egg extracts after cyclin destruction. Biol Cell. 1998;90(8):565–72.
12. Watanabe N, Hunt T, Ikawa Y, Sagata N. Independent inactivation of MPF and cytostatic factor (Mos) upon fertilization of Xenopus eggs. Nature. 1991;352(6332):225–36.
13. Verfaillie MH, Kubik JZ, Clarke HJ, Maro B. Microtubule and chromatin behavior follow MAP kinase activity but not MPF activity during meiosis in mouse oocytes. Development. 1994;120(4):1017–25.
14. Sun QY, Rubinstein S, Breitbart H. MAP kinase activity is downregulated by phorbol ester during mouse oocyte maturation and egg activation in vitro. Mol Reprod Dev. 1999;52(3):310–8.
15. Pokrass MJ, Ryan KA, Xin T, Pielsbick T, Timp W, Greco V, et al. Cell-cycle-dependent ERK signaling dynamics direct fate specification in the mammalian preimplantation embryo. Dev Cell. 2020;55(3):328-340.e325.
16. Patel AL, Shvartsman SY. Outstanding questions in developmental ERK signaling. Development. 2018;145(14):dev143818.
17. Hake SB, Garcia BA, Duncan EM, Kauer M, Dellaire G, Shabanowitz J, et al. Expression patterns and post-translational modifications associated with mammalian histone H3 variants. J Biol Chem. 2006;281(1):559–68.
18. Hake SB, Allis CD. Histone H3 variants and their potential role in indexing mammalian genomes: the “H3 barcode hypothesis.” Proc Natl Acad Sci USA. 2006;103(17):6248–35.
19. Liu H, Kim JM, Aoki F. Regulation of histone H3 lysine 9 methylation in oocytes and early pre-implantation embryos. Development. 2004;131(10):2269–80.
20. Santos F, Peters AH, Otte AP, Reik W, Dean W. Dynamic chromatin modifications characterise the first cell cycle in mouse embryos. Dev Biol. 2005;280(1):225–36.
21. Ma XS, Chao SB, Huang XJ, Lin F, Qin L, Wang XG, et al. The Dynamics and regulatory mechanism of pronuclear H3K9me2 asymmetry in mouse zygotes. Sci Rep. 2015;5:17924.
22. Zeng TB, Han L, Pierce N, Pfeifer GP, Szabo PE. EHMT2 and SETDB1 protect the maternal pronucleus from Smc oxidation. Proc Natl Acad Sci USA. 2019;116(22):10834–41.
23. Shimaji K, Konishi T, Tanaka S, Yoshida H, Kato Y, Ohkawa Y, et al. Genom- 
Chen
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27. Pendina AA, Efimova OA, Fedorova ID, Leont'eva OA, Shilnikova EM,
26. Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, et al. Con-
25. Jones PA. Functions of DNA methylation: islands, start sites, gene bodies
24. Schübeler D. Function and information content of DNA methylation.
2015;20(11):902–14.
23. Shimaji K, Konishi T, Tanaka S, Yoshida H, Kato Y, Ohkawa Y, et al. Genom-
Chen
•

29. Messerschmidt DM, Knowles BB, Solter D. DNA methylation dynamics
during epigenetic reprogramming in the germline and preimplantation 
embryos. Genes Dev. 2014;28(8):812–28.
30. Amouroux R, Nashim B, Shirane K, Nakagawa S, Hill PW, D'Souza Z, et al. De
novo DNA methylation drives Shm accumulation in mammalian 
ygotes. Nat Cell Biol. 2016;18(2):25–35.
31. Nakamura T, Liu YJ, Nakashima H, Umehara H, Inoue K, Matoba S, et al. PGC7 
binds histone H3K9me2 to protect against conversion of 5mC to 
Shm in early embryos. Nature. 2012;486(7403):415–9.
32. Han L, Ren C, Li L, Liu X, Ge J, Wang H, et al. Embryonic defects induced 
by maternal obesity in mice derive from Stella insufficiency in oocytes. 
Nat Genet. 2018;50(3):452–4.
33. Zhao Q, Zhang J, Chen R, Wang L, Li B, Cheng H, et al. Dissecting the 
precise role of H3K9 methylation in crosstalk with DNA maintenance 
methylation in mammals. Nat Commun. 2016;7:12464.
34. Au Yeung WK, Brind'Amour J, Hatano Y, Yamagata K, Feil R, Lorincz MC, 
et al. Histone H3K9 methyltransferase g9a in oocytes is essential for 
preimplantation development but dispensable for CG methylation 
protection. Cell Rep. 2019;27(1):282-293.e284.
35. Frankenberg S, Gerbe F, Bessonard S, Bellville C, Pouchin P, Bardot O, et al. 
Primitive endoderm differentiates via a three-step mechanism involving 
Nanog and RTK signaling. Dev Cell. 2011;21(6):1005–13.
36. Lepikhov K, Walter J. Differential dynamics of histone H3 methylation at 
positions K4 and K9 in the mouse zygote. BMC Dev Biol. 2004;4:12.
37. Smirnogolpithak N, Sundriyal S, Li F, Vedadi M, Fuchter MJ. Identifi-
cation of 2,4-diamino-6,7-dimethoxyquinoline derivatives as G9a 
histone methyltransferase. Mol Cell. 2007;25(3):473–81.
38. Kubicke S, O'Sullivan RJ, August EM, Hickey ER, Zhang Q, Teodorro ML, 
et al. Reversal of H3K9me2 by a small-molecule inhibitor for the G9a 
histone methyltransferase. Mol Cell. 2007;25(3):473–81.
39. Smith ZD, Chan MM, Mikkelsen TS, Gu H, Gnrke A, Regev A, et al. A 
unique regulatory phase of DNA methylation in the early mammalian 
embryo. Nature. 2012;484(7394):339–44.
40. Guo F, Li X, Liang G, Li T, Zhu P, Guo H, et al. Active and passive demeth-
ylation of male and female pronuclear DNA in the mammalian zygote. 
Cell Stem Cell. 2014;15(4):447–59.
41. Shem L, Inoue A, He J, Liu Y, Lu F, Zhang Y Tet3 and DNA replication medi-
ate demethylation of both the maternal and paternal genomes in mouse 
ygotes. Cell Stem Cell. 2014;15(4):459–71.
42. Rice JC, Briggs SD, Ueberheide B, Barber CM, Shabanowitz J, Hunt DF, 
et al. Histone methyltransferases direct different degrees of methylation 
to define distinct chromatin domains. Mol Cell. 2003;12(6):1591–8.
43. Maison C, Aloumizi G. HP1 and the dynamics of heterochromatin main-
tenance. Nat Rev Mol Cell Biol. 2004;5(4):296–304.
44. Hamilton WB, Mosesson Y, Monteiro RS, Erdal KB, Knudsen TE, Francavilla 
c, et al. Dynamic lineage priming is driven via direct enhancer regulation 
by ERK. Nature. 2019;575(7782):355–60.
45. Wu BK, Brenner C. Suppression of TET1-dependent DNA demethylation is 
essential for KRAS-mediated transformation. Cell Rep. 2014;9(5):1827–40.
46. Gu TP, Guo F, Yang H, Wu HP, Xu G, Liu W, et al. The role of Tet3 DNA 
dioxigenase in epigenetic reprogramming by oocytes. Nature. 2011;477(7366):606–10.
47. Zylciz J, Borenstein M, Wong FC, Huang Y, Lee C, Dietmann S, et al. G9a 
regulates temporal preimplantation developmental program and lineage 
segregation in blastocyst. Elife. 2018;7:e33631.
48. Huang B, Chen J, Shen J, Li W, Liu L, Li B, et al. Stella safeguards the oocyte 
methylome by preventing de novo methylation mediated by DNMT1. 
Nature. 2018;564(7734):136–40.
49. Han L, Ren C, Zhang J, Shu W, Wang Q. Differential roles of Stella in the 
modulation of DNA methylation during oocyte and zygotic development. 
Cell Discov. 2019;5:9.
50. Kristensen DG, Skakkebaek NE, Rajpert-De Meyts E, Altmstrup K. Epigenetic 
features of testicular germ cell tumours in relation to epigenetic character-
istics of foetal germ cells. Int J Dev Biol. 2013;57(2–4):309–17.
51. Girnán-Ferré C, Marsal-García L, Belfver-Sanchis A, Kondengaden SM, 
Turga RC, Vázquez S, et al. Pharmacological inhibition of G9a/GLP 
restores cognition and reduces oxidative stress, neuroinflammation and 
β-Amyloid plaques in an early-onset Alzheimer's disease mouse model. 
Aging. 2019;11(23):11591–608.

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