Maternal Sall4 Is Indispensable for Epigenetic Maturation of Mouse Oocytes

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Sall4 (Splat-like 4) plays important roles in maintaining pluripotency of embryonic stem cells and in various developmental processes. Here, we find that Sall4 is highly expressed in oocytes and early embryos. To investigate the roles of SALL4 in oogenesis, we generated Sall4 maternal specific knock-out mice by using CRISPR/Cas9 system, and we find that the maternal deletion of Sall4 causes developmental arrest of oocytes at germinal vesicle stage with non-surrounded nucleus, and the subsequent meiosis resumption is prohibited. We further discover that the loss of maternal Sall4 causes failure in establishment of DNA methylation in oocytes. Furthermore, we find that Sall4 modulates H3K4me3 and H3K27me3 modifications by regulating the expression of key histone demethylases coding genes Kdm5b, Kdm6a, and Kdm6b in oocytes. Moreover, we demonstrate that the aberrant H3K4me3 and H3K27me3 cause mis-expression of genes that are critical for oocytes maturation and meiosis resumption. Taken together, our study explores a pivotal role of Sall4 in regulating epigenetic maturation of mouse oocytes.

In mammals, oocyte maturation is an important developmental process, which is prerequisite for the subsequent fertilization and embryo development. In mice, oocytes reside in the ovarian follicles, and follicles can be divided into five stages according to the developmental process: primordial follicle, primary follicle, secondary follicle, early antral follicle, and antral follicle (1). During this process, the oocyte will undergo maturation as the follicle grows into antral follicle. Then the mature oocyte acquires the ability to resume meiosis with its nucleus forming surrounded nuclear (SN) configuration and the transcription quiescence occurring simultaneously.

The oocyte maturation encompasses the following three main processes: nuclear maturation, cytoplasmic maturation, and epigenetic maturation. Comparing with the nuclear and cytoplasmic maturation, the underlying mechanism of oocyte epigenetic maturation is not fully understood. Previous studies have indicated that DNA methylation and histone modifications play functional roles in oocytes maturation. De novo DNA methylation starts to occur in the secondary follicle oocytes and completes when oocytes acquire the competence of resuming meiosis (2). Among all the DNA methyltransferases (DNMTs), DNMT3A and DNMT3L are mainly responsible for the establishment of DNA methylome in oocytes (3). Meanwhile, as another important epigenetic modification, histone modifications have been demonstrated important for chromosome organization, chromosome segregation, and meiotic resumption during oocytes maturation (4). However, it remains elusive how histone modifications are regulated and to what extent the transcriptome is influenced during oocytes maturation.

SALL4, as a zinc finger protein, was first identified in Drosophila. In humans, SALL4 mutations cause Okihiro syndrome, with multiple organs having developmental abnormalities (5). In mice, Sall4 is primarily expressed in early embryos, embryonic stem cells (ESCs), primordial germ cells, and germ cells with different and specific functions. Sall4 null embryos die shortly after implantation on embryonic day 6.5 (6). In ESCs, Sall4 can activate the pluripotent master gene Pou5f1 (7) and recruit nucleosome remodeling and deacetylase (NuRD) com-

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The abbreviations used are: SN, surrounded nuclear; DNMT, DNA methyltransferase; ESC, embryonic stem cell; NuRD, nucleosome remodeling and deacetylase; qRT-PCR, quantitative real time PCR; IF, immunofluorescence; GVBD, germinal vesicle breakdown; Smc, 5-methylcytosine; UHPLC-MS/MS, ultra high performance liquid chromatography-electrospray ionization-quadrupole mass spectrometry; RRBS, reduced representation bisulfite sequencing; SF, secondary follicle; EAF, early antral follicle; gDMR, germ cell differentially methylated region; Pn, postnatal day n.
plex to suppress the trophoderm marker Cdx2 (8). Other studies have shown that SALL4 acts as an epigenetic regulator in ESCs by recruiting DNMTs, HDAC1, and HDAC2 to methylate CpG islands and deacetylate the histone tails in active chromatin regions (9). In germ cell development, Sall4 plays essential roles in ensuring the correct specification and migration of primordial germ cells (10). In male mice, SALL4 interacts with PLZF and promotes the specification of spermatogonial progenitor cells (11). However, whether Sall4 functions in oogenesis remains unknown.

In the present study, we aimed to investigate whether the maternal Sall4 plays a role in oocyte maturation and subsequent totipotency establishment. We first confirmed that Sall4 is highly expressed in oocytes at different developmental stages. Then we investigated the function and mechanism of SALL4 in oogenesis by specifically deleting Sall4 in oocytes. Our results indicate that maternal SALL4 functions as an epigenetic modulator and plays an essential role in the epigenetic maturation of oocytes.

Results

Characterization of SALL4 in Oogenesis and Preimplantation Embryo Development—We first identified the expression pattern of Sall4 in oogenesis by conducting quantitative real time PCR (qRT-PCR) and immunofluorescence (IF) staining. Sall4 expression begins in primary follicle stage oocytes and continues accumulating as the oocytes grow (Fig. 1, A and B). During this period, SALL4 was localized in the nucleus (Fig. 1B). When germinal vesicle breakdown (GVBD) occurs, SALL4 diffuses into the cytoplasm (Fig. 1E, MII). After fertilization, SALL4 aggregates in the pronuclei but dramatically degenerates during the first cleavage. Then SALL4 re-expressed and accumulated until the formation of blastocyst (Fig. 1, D and E). The live cell imaging of oocytes collected from Sall4-mCherry transgenic mice further confirmed this expression and localization pattern of SALL4 (Fig. 1, C and F). These results imply that SALL4 may be a maternal factor and play important roles in oogenesis and preimplantation embryo development.

Maternal Sall4 Knock-out Oocytes Are Immature—To identify the effects of maternal SALL4 in oogenesis, we first generated Sall4<sup>fl/fl</sup> mice using CRISPR/Cas9 system. By crossing with Zp3-Cre or Gdf9-Cre transgenic mice, we then obtained Sall4<sup>fl/fl;Zp3-Cre</sup> and Sall4<sup>fl/fl;Gdf9-Cre</sup> female mice. However, Sall4<sup>fl/fl;Zp3-Cre</sup> and Sall4<sup>fl/fl;Gdf9-Cre</sup> female mice were infertile (Table 1). Hematoxylin and eosin staining indicated that antral follicles were absent in ovaries of both genotypes (Fig. 2, A and F). Apart from histological results, the non-SN conformation and high
transcription activity in SALL4 null oocytes further indicated that they were immature (Fig. 3, B and C). Moreover, to identify whether SALL4 null oocytes were partially competent to resume meiosis, we co-cultured the WT or SALL4 null oocytes with WT granulosa cells in vitro for 16 h. The results showed that SALL4 null oocytes could not undergo GVBD (Fig. 3, D and E), indicating that SALL4 null oocytes were not even partially mature.

**SALL4 Is Essential for De Novo DNA Methylation by Interacting with DNMT3A**—To identify the mechanism of SALL4 in oocyte maturation, we first focused on the factors interacting with SALL4. IF staining showed that the contents and localization of NuRD complex core components and DNMT3B were not altered in SALL4 null oocytes (supplemental Fig. S1, A–C). Surprisingly, the nuclear localization of DNMT3A was obviously lost in SALL4 null oocytes (Fig. 4A), and the IF staining for 5-methylcytosine (5mC) showed that the SALL4 null oocytes were hypomethylated (Fig. 4B). Furthermore, using an ultra-sensitive ultra high performance liquid chromatography-electrospray ionization-quadrupole mass spectrometry (UHPLC-MS/MS) approach for absolute quantification analysis of 5mC demonstrated that the DNA methylation levels in SALL4 null oocytes was ~75% lower than WT oocytes (Fig. 4C). To obtain a detailed DNA methylation profile of SALL4 null oocytes, we performed reduced representation bisulfite sequencing (RRBS) using secondary follicle (SF) stage and early antral follicle (EAF) stage oocytes from Sall4fl/+/Zp3-Cre and Sall4fl/fl;Zp3-Cre mice separately. The result showed that the whole genome of SALL4 null oocytes were extensively hypomethylated. Moreover, the maternal germ cell differentially methylated regions (gDMRs) and imprinting control regions were barely methylated. In addition, the repeated elements were also hypomethylated (Fig. 4D). The bisulfite sequencing PCR on maternal gDMRs (Ifg2r and Mst2) and repetitive sequence regions (Line1 and LAR-LTR) further confirmed the results of RRBS analysis (Fig. 4E). Therefore, the loss of SALL4 can cause de novo DNA methylation failure probably by influencing the nuclear deposition of DNMT3A.

The Transcriptome of SALL4 Null Oocytes Is Dramatically Perturbed—To further verify why SALL4 null oocytes cannot undergo maturation, we performed single-cell RNA sequencing on SF and EAF stage oocytes from Sall4fl/fl;Zp3-Cre and Sall4fl/fl mice, respectively. The RNA-Seq results showed that more than 4,000 genes were mis-expressed in SALL4 null oocytes (Fig. 5, A and B). Specifically, SALL4 null SF and EAF stage oocytes showed 2,230 down-regulated genes (with 973 genes overlapped) and 2,030 up-regulated genes (with 738 genes overlapped) (Fig. 5, C and D). Then we conducted Gene Ontology analysis on the overlapped differentially expressed genes. There was a lot of phosphorylation, and oxidative stress response-related genes were highly expressed in SALL4 null oocytes. The transmission electron microscopy analysis also showed that the SALL4 null oocytes have much thinner zona pellucida, abnormal mitochondria, and endoplasmic reticulum (supplemental Fig. S2A), whereas the down-regulated genes in SALL4 null oocytes were mainly chromosome organization-, transcription regulation-, and cell cycle-related (Fig. 5E). Above all, SALL4 null oocytes showed obvious disorders in metabolism, transcriptome, and epigenome. Moreover, the aberrant expression of cell cycle-related genes can partially explain why SALL4 null oocytes cannot undergo GVBD. We then analyzed the correlation between the transcriptome disorders and DNA methylome abnormalities by comparing gene expression levels and methylation levels of gene promoter regions. In SALL4 null oocytes, the hypomethylated or hypermethylated genes relative to WT oocytes showed no correlation with gene expression levels (Fig. 5F), which indicates that DNA methylation cannot explain the transcriptome disorders in SALL4 null oocytes.

**SALL4 Modulates H3K4me3 and H3K27me3 by Regulating IAP**—In view of the extensive and dramatic alteration of transcriptome in SALL4 null oocytes, we inferred that SALL4 might regulate transcription through modulating some other epigenetic modifications. Therefore, we combined the published SALL4 ChIP-Seq data (GSE73390) (12) with our RNA-Seq data to screen histone modification-
related genes, which are regulated by SALL4. Then we focused on several histone lysine demethylase coding genes: Kdm5b, Kdm6a, and Kdm6b. In detail, the abnormal high expression level of Kdm5b, as well as the low expression levels of Kdm6a and Kdm6b in SALL4 null oocytes, were confirmed by qRT-PCR (Fig. 6A). In addition, ChIP-Seq data and luciferase reporter assays showed SALL4 bound primarily at the promoter of these genes (Fig. 6B and supplemental Fig. S2B). Correspondingly, the level of H3K4me3 was lower, and the level of H3K27me3 was higher in SALL4 null oocytes compared with WT oocytes (Fig. 6C and D, and supplemental Fig. S3A). Thus, we hypothesized that the abnormalities of H3K4me3 and H3K27me3 levels might account for the transcriptome disorders in SALL4 null oocytes. To validate this hypothesis, we then injected Kdm5b mRNA and siRNAs targeting Kdm6a and Kdm6b together into postnatal day 10 (P10) WT oocytes, which was set as the experimental group. For control group oocytes, GFP mRNA and scramble siRNAs were injected. IF staining results demonstrated that the oocytes in experimental group could mimic the changes of H3K4me3 and H3K27me3 observed in SALL4 null oocytes (Fig. 7A). After in vitro culture and maturation induction, the GVBD rate of oocytes was calculated. The results showed that /H1101170% oocytes could undergo GVBD in control group, whereas the GVBD rate in experimental group was only 25% (Fig. 7B and C). Moreover, RNA-Seq analysis was conducted on oocytes randomly collected from both experimental and control group separately. Then we analyzed the functions of overlapped mis-expressed genes in both the experimental group oocytes and the SALL4 null oocytes, and found that the overlapped mis-expressed genes were mainly related to responses of hormone stimulations and cell surface-linked signal transduction (Fig. 7D). Among these genes, glial cell-derived neurotrophic factor family receptor alpha1 (Gfra1) was well studied in oogenesis. During oocytes

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FIGURE 3. SALL4 null oocytes are immature. A, hematoxylin and eosin (H&E) and SALL4 immunohistochemistry staining in P21.5 WT and two kinds of knock-out mice ovaries. Scale bars, 100 μm. B, the SN/non-SN (NSN) rate of oocytes in P21.5 WT and Sall4flfl;Zp3-Cre (hereinafter referred to as KO) mice. The data represent the means ± S.E. (n = 3). C, BrUTP immunofluorescence staining results of WT and KO mice. The left two panels showed oocytes before in vitro culture, and the right two panels showed oocytes after maturation induction. The WT oocytes showed GVBD obviously and polar bodies could be seen in parts of oocytes, whereas the germinal vesicles remained in KO oocytes. The polar bodies were pointed by red arrows. Scale bars, 50 μm. D, morphology of oocytes derived from WT and KO mice after in vitro maturation. Each experiment was conducted in triplicate. The data represent the means ± S.E. (n = 3). E, GVBD rate of WT and KO oocytes after in vitro maturation. Each experiment was conducted in triplicate. The data represent the means ± S.E. (n = 3). F, numbers of follicles of indicated stages in Sall4flfl, Sall4flfl;Zp3-Cre and Sall4flfl;Gdf9-Cre mice at P22.5 (46 h after treatment of PMSG). The data represent the means ± S.E. (n = 3).

TABLE 1 Both Sall4flfl;Zp3-Cre and Sall4flfl;Gdf9-Cre were infertile

|              | Sall4flfl | Sall4flfl;Zp3-Cre | Sall4flfl;Gdf9-Cre |
|--------------|-----------|-------------------|-------------------|
| Number of breedings | 15        | 0                 | 0                 |
| Total number of pups | 133       | 0                 | 0                 |
| Pups per breeding | 8.87      | 0                 | 0                 |

injected Kdm5b mRNA and siRNAs targeting Kdm6a and Kdm6b together into postnatal day 10 (P10) WT oocytes, which was set as the experimental group. For control group oocytes, GFP mRNA and scramble siRNAs were injected. IF staining results demonstrated that the oocytes in experimental group could mimic the changes of H3K4me3 and H3K27me3 observed in SALL4 null oocytes (Fig. 7A). After in vitro culture and maturation induction, the GVBD rate of oocytes was calculated. The results showed that ~70% oocytes could undergo GVBD in control group, whereas the GVBD rate in experimental group was only 25% (Fig. 7B and C). Moreover, RNA-Seq analysis was conducted on oocytes randomly collected from both experimental and control group separately. Then we analyzed the functions of overlapped mis-expressed genes in both the experimental group oocytes and the SALL4 null oocytes, and found that the overlapped mis-expressed genes were mainly related to responses of hormone stimulations and cell surface-linked signal transduction (Fig. 7D). Among these genes, glial cell-derived neurotrophic factor family receptor alpha1 (Gfra1) was well studied in oogenesis. During oocytes
maturation, human chorionic gonadotropin stimulates the granulosa cells to secret glial cell-derived neurotrophic factors, which further induce the oocytes to grow and mature (13, 14). Loss of function experiments have also proved the essential roles of Gfra1 in successful fertilization of oocytes (15). Moreover, the platelet derived growth factor Pdgfa and prolactin receptor Prlr have been reported to be essential for oocyte maturation by their functions in oocytes-granulosa cells interactions (16, 17). In addition, the down-regulation of mechanistic target of rapamycin Mtor and homeobox A7 Hoxa7 has been shown to be detrimental to oocyte growth (18, 19). To further verify whether these oogenesis related genes are regulated by H3K4me3 and H3K27me3 levels, we performed ChIP-qPCR assays. Because of the shortage of oocytes, we applied ultra low input ChIP-qPCR to investigate the levels of H3K4me3 and H3K27me3 on the promoter regions of Gfra1, Pdgfa, Prlr, Mtor, and Hoxa7. The results showed that the H3K4me3 levels on the promoters of these genes in SALL4 null oocytes are much lower than in the WT oocytes (Fig. 7E, upper panel), and the H3K27me3 levels on the promoters of these genes in SALL4 null oocytes are much higher than in the WT oocytes (Fig. 7E, lower panel). Therefore, we can conclude that H3K4me3 and H3K27me3 regulate the expression of Gfra1, Pdgfa, Prlr, Mtor, and Hoxa7 in oocytes. Above all, we verified that the proper levels of H3K4me3 and H3K27me3 guard the normal transcriptome, which are critical for oocyte-granulosa cell interactions and oocyte growth.

Discussion

Recent studies have revealed DNA regions with non-methylated H3K4 and tri-methylated H3K36 are preferentially methylated (20, 21). However, although the histone modifications in SALL4 null oocytes (low levels of H3K4me3 and high levels of H3K36me3 (Fig. 6E and supplemental Fig. S3A) are propitious for DNA methylation establishment, without SALL4, the de novo DNA methylation still failed. Such contrary results have also been found in HDAC1/2 knock-out oocytes (22, 23). Thus, de novo DNA methylation may also rely on specific factors apart from histone modifications. Furthermore, SALL4, HDAC1, and HDAC2 are all related to NuRD complex, which prompts us to put forward a hypothesis that NuRD complex may take parts in de novo DNA methylation during oogenesis.

In the microinjection experiment, there were still 25% of oocytes that could undergo GVBD. One possible reason was that the follicles used for microinjection were obtained from P10.5 mice; however, SALL4 depletion occurred at P6.5 or earlier in Sall4fl/fl;Zp3-Cre mice. In addition, SALL4, as an important transcription factor, definitely can directly regulate other genes which play roles in oocyte maturation, even though the microinjection experiment still powerfully validated that the proper levels of H3K4me3 and H3K27me3 were important for regulating transcriptome in oogenesis and were essential for oocytes maturation. Because of limited materials, previous studies mainly focused on how histone marks influenced the nuclear conformation, which are convenient to observe. How-
ever, how histone marks modulated transcriptome in oogenesis was less studied. In our study, we interpreted how H3K4me3 and H3K27me3 modulated transcription of certain genes that were essential for oocytes maturation by oocytes microinjection, single-cell RNA-Seq assays and ultra low input ChIP-qPCR.

In summary, we found that oocyte-specific Sall4 knockout mice showed severe defects in oogenesis including impaired follicle development and meiosis resumption inhibition. We demonstrated that SALL4 null oocytes showed a severely abnormal transcriptome and aberrant epigenome including failure in DNA methylation establishment and histone modifications abnormalities. As for DNA methylation, SALL4 was indispensable for the nuclear localization of DNMT3A and thus essential for DNA methylation establishment in the process of oocytes maturation. As for histone modifications, SALL4 regulated the expression of Kdm5b, Kdm6a, and Kdm6b, which then modulated the levels of H3K4me3 and H3K27me3. In turn, the abnormal H3K4me3 and H3K27me3 modifications led to mis-expression of many key genes essential for oocytes maturation (supplemental Fig. S3B). Overall, our present study elucidated a pivotal role of pluripotency factor, Sall4, in epigenetic maturation of mouse oocytes.

**Experimental Procedures**

*Mice Generation and Maintenance—Sall4<sup>fl/fl</sup> mice and Sall4-mCherry mice were generated using CRISPR/Cas9 system. All experiments were performed in accordance with the University of Health Guide for the Care and Use of Laboratory Animals.*
and were approved by the Biological Research Ethics Committee of Tongji University.

Oocytes and Early Embryo Collection and Culture—Primordial, primary, secondary, early antral, and antral follicles were obtained from Sall4fl/fl;Zp3-Cre female mice at P2.5, P6.5, P12.5, P17.5, and P22.5 as previously described (24). Fully grown oocytes were isolated from 4–6-week-old mice 46 h after PMSG injection. Zygotes were obtained from the ampulla of the uterine tube of superovulated female mice after mating with male mice. Then two-cell, four-cell, eight-cell, morula, and blastocyst embryos were obtained by culturing zygotes in Quinn’s Advantage medium (In Vitro Fertilization, Inc.).

SN/GVBD Ratio Calculation and Statistics—For SN/non-SN ratio calculation, we obtained oocytes from P21.5 Sall4fl/fl and Sall4fl/fl;Zp3-Cre female mice. Then we use Hoechst 33258 (Thermo Fisher Scientific) to stain the nucleus DNA. Then we observed the nucleus of oocytes under fluorescence microscope and counted the oocytes in SN or non-SN conformation. Three independent experiments were performed for each WT and KO groups. For GVBD ratio calculation, we obtained oocytes from P21.5 Sall4fl/fl and Sall4fl/fl;Zp3-Cre female mice and then cultured these oocytes in vitro for 16 h. Then we observed the germinal vesicles of each oocyte. Then we counted the amount of oocytes with or without germinal vesicles. The experiments were conducted for three times independently.

Immunofluorescent Staining—For immunofluorescent staining, collected oocytes and embryos were fixed in 4% paraformaldehyde for 15 min and then permeabilized for 15 min in 0.3% Triton X-100. For 5mC staining, an additional 30 min of 4 N HCl treatment and three washes in Tris (pH 8.0) were needed. The samples were blocked in PBS with 2.5% BSA. Then they were incubated with the primary antibodies. Next, the samples were washed and incubated with secondary antibodies. After being washed in PBS and incubated with DAPI, the samples were observed under confocal microscope.

BrUTP Incorporation Assay—Oocytes obtained from P21.5 Sall4fl/fl or Sall4fl/fl;Zp3-Cre mice were injected with 5 mM BrUTP (Sigma). Then the oocytes were washed three times and...
cultured in the incubator (37 °C, 5%CO₂). 25 min later, the oocytes were fixed and proceeded to IF staining for BrUTP.

**Bisulfite Sequencing PCR**—Approximately 200 EAF stage WT or KO oocytes were used for genomic DNA isolation with the QIAamp DNA Micro Kit (Qiagen). Then DNA was treated with the MethylCode bisulfite conversion kit (Invitrogen). Next, EpiTect whole bisulfitome kit (Qiagen) was used to amplify the converted genome. Then nested PCRs was performed to amplify the gDMR regions of the indicated genes. The amplified products were cloned into vectors with the pEASY™-T5 Zero cloning kit (TransGen Biotech), 10–16 randomly selected clones were sequenced in Genewiz, Inc. Primers used in this analysis are listed in Table 2.

**UHPLC-MRM-QQQ Analysis for Oocytes**—Sample preparation prior to the UHPLC-MS/MS analysis was operated as described previously (25). The analysis was performed on an Agilent 1290 Infinity ultrahigh performance LC system coupled with an Agilent QQQ6490 mass spectrometer equipped with a jet stream electrospray ionization source (Santa Clara). The mass spectrometer was operated under positive ionization with multiple reactions monitoring mode.

**Oocytes Microinjection and in Vitro Maturation**—We detached secondary follicles from P10.5 WT female mice ovaries. We randomly separated the follicles into two groups, and then we injected siRNAs and mRNAs into the oocytes using microinjection facilities. Next, the follicles were cultured in

**FIGURE 7.** Proper levels of H3K4me3 and H3K27me3 are essential for oocyte maturation. A, IF staining for H3K4me3 and H3K27me3 of oocytes after injection manipulation for 7 days in control (Ctrl) and experimental (Exp) groups. Scale bars, 10 μm. B, morphology of oocytes in control and experimental groups after maturation induction for 24 h. Scale bars, 50 μm. The oocytes in the control group underwent GVBD and polar bodies were obvious in some oocytes, whereas the germinal vesicles remained in most of the oocytes in the experimental group. C, statistics analysis of GVBD rate in control groups and experimental groups. In total, 313 oocytes were injected in control groups and 332 oocytes were injected in experimental groups. The data represent the means ± S.E. (n = 3). ***p < 0.001, Student’s t test. D, heat map of typically differentially expressed genes in all kinds of KO (or experimental groups) oocytes. E, ChIP-qPCR analysis of H3K4me3 and H3K27me3 levels on the promoter regions of oogenesis key genes. The upper panel showed the H3K4me3 enrichment levels on the promoter regions. The lower panel showed the H3K27me3 enrichment levels on the promoter regions. All enrichment values are relative to each input enrichment values and then normalized with WT enrichment values. The data represent the means ± S.E. (n = 6 = 2 ChIP replicates × 3 qPCR replicates). ***p < 0.001; *, p < 0.05, Student’s t test.
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**TABLE 2**

| Primers for qRT-PCR, bisulfite sequencing, and ChiP-qPCR primers |
|---|
| **Name** | **Sequence** |
| Kdm6a | CCGGGTTGACCAAAAGGAGGAC |
| Reverse | GCTGACGACACTGGTGCAC |
| Kdm5b | AGCGGGAACCTGGTGTACAA |
| Reverse | GAGGGGAAGCTGTCGGTCTCC |
| Mest | TGAGAGCATCCTGAGGTTT |
| Reverse | TCTGCCTGCTGACAGT |
| Igf2r | TTATGGGGTTATTTTTTTAGTTG |
| Reverse | AATGATCTCTCTCCATCTCATCT |
| H19 | GTAAGAGATTTTATGATTTTTTTAG |
| Reverse | ACTGATATTTTTCTCACAACCT |
| Line 1-forward | TCTGATTTTGGATATAAAAGGTTAATGAG |
| Reverse | TTTTGGATATTGAGGGTTTTAGGTTG |
| Lap-LTR-forward | AAATATCCTAAAAATACAAACTACAC |
| Reverse | GTGTGGTATTTTTATGTATAGTTAGG |
| Gfra1 | ATGAGTATTTAGGAGGTATAAGAATT |
| Reverse | AGAGCTTGAAACAGGTAGCCGA |

by a reverse transcription system using 5× All-In-One RT MasterMix (ABM). Quantitative RT-PCR was performed using SYBR Green master mix (Vazyme, Nanjing, China). The primers used are shown in Table 2. The primers were synthesized at Genewiz, Inc.

**RNA-Seq and RRBS Analysis**—All RNA-Seq reads were mapped and quantified as previously described (30, 31). The number of mapped reads was counted using htsq-cound (v 0.6.0) (32). Differential expression analysis was conducted by edgeR (v 3.10.2) using read counts. Genes with a Benjamini and Hochberg-adjusted p value (false discovery rate) < 0.05 and a mean fold change of > 1 were termed differentially expressed. Among all the RNA-Seq data, the SF WT group has two replicates; the SF KO group, the EAF WT group, and the EAF KO group all have three replicates. All the RRBS sequencing reads were mapped as previously described (33, 34). The methylation level of each CpG site was estimated using mcall (v 1.3.0) with default parameters, and CpG sites with read depths ≥ 1 were counted as total CpG coverage of the sample. The bisulfite conversion ratio for each sample was calculated using mcall (v 1.3.0) with default parameters, and CpG sites with read depths ≥ 1 were counted as total CpG coverage of the sample. The bisulfite conversion ratio for each sample was calculated using mcall (v 1.3.0) with default parameters, and CpG sites with read depths ≥ 1 were counted as total CpG coverage of the sample. The bisulfite conversion ratio for each sample was calculated using mcall (v 1.3.0) with default parameters, and CpG sites with read depths ≥ 1 were counted as total CpG coverage of the sample.

**Ultra Low Input ChiP-qPCR**—For ultra low input ChiP-qPCR, 600 oocytes were used per reaction. All oocytes were washed three times in 0.5% BSA-PBS (Sigma) solution to avoid any possible contamination. The procedure of ULI-ChiP was carried out as previously described (35). 1 μg of histone H3K4me3 antibody (Cell Signaling Technology, catalog no. 9727) or 1 μg of histone H3K27me3 antibody (Diagnose, catalog no. pAb-069-050) was used for each immunoprecipitation reaction. Then we used 3 ng/μl DNA obtained from the ChiP experiments and 10 ng/μl input DNA for ChiP-qPCR analysis. The primers used in the qPCR experiment are listed in Table 2. There are two replicates each for the H3K4me3 WT group, the H3K4me3 KO group, the H3K27me3 WT group, and the H3K27me3 KO group.

**Author Contributions**—K. X. and X. C. performed most of the experiments. H. Y., X. X., Y. H., C. W., B. L., W. L., J. Y. L., X. K., Y. Z., K. Z., L. Z., H. W., J. L., H. F., F. W., Y. G., and Y. Z. helped with experiments and data analysis. K. X., X. C., J. C., and S. G. designed the research, analyzed data, and wrote the paper.

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