MLL2 Is Required in Oocytes for Bulk Histone 3 Lysine 4 Trimethylation and Transcriptional Silencing

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Abstract

During gametogenesis and pre-implantation development, the mammalian epigenome is reprogrammed to establish pluripotency in the epiblast. Here we show that the histone 3 lysine 4 (H3K4) methyltransferase, MLL2, controls most of the promoter-specific chromatin modification, H3K4me3, during oogenesis and early development. Using conditional knockout mutagenesis and a hypomorph model, we show that MLL2 deficiency in oocytes results in anovulation and oocyte death, with increased transcription of p53, apoptotic factors, and lap elements. MLL2 is required for (1) bulk H3K4me3 but not H3K4me1, indicating that MLL2 controls most promoters but monomethylation is regulated by a different H3K4 methyltransferase; (2) the global transcriptional silencing that precedes resumption of meiosis but not for the concomitant nuclear reorganization into the surrounded nucleolus (SN) chromatin configuration; (3) oocyte survival; and (4) normal zygotic genome activation. These results reveal that MLL2 is autonomously required in oocytes for fertility and imply that MLL2 contributes to the epigenetic reprogramming that takes place before fertilization. We propose that once this task has been accomplished, MLL2 is not required until gastrulation and that other methyltransferases are responsible for bulk H3K4me3, thereby revealing an unexpected epigenetic control switch amongst the H3K4 methyltransferases during development.

Introduction

Mammalian epigenomes are fundamentally reprogrammed during gametogenesis and pre-implantation development to establish the ground state of pluripotency in the epiblast cells of the blastocyst [1–5]. Despite the importance of this epigenetic reprogramming, how such changes are achieved is not well understood. Epigenetic reprogramming of the maternal genome occurs during oogenesis. Oocytes develop synchronously from birth until puberty, during which time they are arrested in meiotic prophase I, increase in size, and are transcriptionally active until the peri-ovulatory stage, when they undergo global transcriptional silencing [6,7]. Transcription of the oocyte genome serves to establish the reservoirs of maternal components that are required for the first stages of embryonic development [8]. Global transcriptional silencing in oocytes is thought to be required for the efficient resumption and completion of meiosis [9] and occurs parallel to large-scale chromatin condensation and rearrangement around the nucleolus to establish a chromatin configuration termed SN (surrounded nucleolus) [1,10,11]. Previous studies showed that global transcriptional silencing can occur without the establishment of the SN state [1,12]. Further studies indicated that both the acquisition of the SN configuration and transcriptional silencing are pre-requisites to achieve full embryonic developmental potential [13,14].

Chromatin and epigenetic changes during oocyte development include histone variant exchange, alterations of DNA methylation, and global shifts in histone post-translational modifications. For instance, maternal-specific genomic imprints are established on a locus-by-locus basis [15–19], the linker histone 1 variant H1FOO is incorporated into chromatin [20,21], and the global levels of 5-methyl-cytosine (5mC DNA methylation) and histone 4 acetylation at lysine 5 and 12 (H4K5 and H4K12) increase [22]. In addition, the levels of di- and tri-methylation of histone 3 at lysines
Author Summary

It is well established that gametes and early mammalian embryos undergo extensive epigenetic changes, which are changes in phenotype or gene expression that do not entail changes in DNA sequence. However, the machinery responsible for epigenetic modification in these situations is poorly understood. In mice, we conditionally deleted the histone 3 lysine 4 (H3K4) methyltransferase Mll2, an enzyme that alters DNA structure and packaging, either in gametes or in somatic cells of the ovary and also produced a mouse hypomorph expressing low levels of Mll2. We show that Mll2 is required in oocytes during gametogenesis and is also needed as a maternally derived factor during early development. Oocytes deficient in Mll2 display decreased methylation of H3K4 (H3K4me3) and show abnormal maturation and gene expression, in particular of pro-apoptotic factors. In addition, we demonstrate that embryonic genome activation is compromised in the absence of Mll2. Together our results identify Mll2 as one of the key players in the epigenetic reprogramming required for female fertility in the mouse.

Results

Oocyte and Granulosa Cell-Specific Conditional Knockouts

The epigenome in the female promonucleus of zygotes is established in oocytes [29], under both autonomous and ovarian control [14]. We first evaluated Mll2 expression in ovarian cells and during embryogenesis by PCR analysis. The results show that both ovarian granulosa cells and oocytes from wild type (WT) females express Mll2 (Figure 1A). QPCR analysis of meiotically incompetent (immature) and peri-ovulatory oocytes revealed a 5-fold increase in Mll2 expression as oocytes approach ovulation, suggesting a potential role for Mll2 during fertilization and/or early embryogenesis (Figure 1B; n = 3; Student’s t test; p<0.05). Mll2 mRNA was present in 1-cell embryos, likely as a maternal product, and it was expressed from the 2-cell stage to the blastocyst stage as determined by QPCR analysis (Figure 1C).

We used a floxed conditional allele (Mll2flox) to dissect Mll2 function (Figure 1D). Previous studies with this allele and a tamoxifen-inducible Cre mouse line showed that near-ubiquitous loss of Mll2 in adult males led to sterility [39]. Here we show that near-ubiquitous loss of Mll2 in adult females also leads to sterility (Figure S1), further suggesting roles for Mll2 in gametogenesis. Because Mll2 is expressed in both granulosa cells and oocytes, we used cell-type specific conditional mutagenesis of the conditional Mll2 allele. Conditional knockout (cKO) mice were generated using Cre recombinase driven by the growth differentiation factor 9 (Gdf9) and zona pellucida (Zp3) promoters (Figure 1E, which are expressed in oocytes [40,41]) or by the anti-Mullerian hormone receptor 2 (Amhr2) promoter, which is expressed primarily in ovarian granulosa cells in adult females [42]. It is noteworthy that expression of Gdf9-Cre in the oocyte occurs in all follicular stages from postnatal day 3, whereas expression of Zp3-Cre begins at postnatal day 5 from the primary follicular stage onwards [40].

Mll2 Deletion Mediated by Gdf9-Cre Results in Female Sterility and Premature Ovarian Follicle Loss

The effect of Cre recombination on Mll2 expression in oocytes was examined by QPCR analysis. Oocytes from Mll2flox/Gdf9-Cre females (herein called Mll2flox/cKO) displayed an 80% decrease in Mll2 mRNA expression compared to WT controls (Figure 1F). Cre recombination deletes exon 2 causing a frame-
A  *MII2* expression in ovarian cells

B  *MII2* expression in oocyte stages

C  *MII2* expression in embryogenesis

D  *MII2* alleles

E  Breeding scheme

F  *MII2* mRNA in cKO oocytes

G  MLL2 protein in cKO oocytes

H  Fertility studies
shift and thus no functional protein is produced. Comcomitantly, mRNA levels fall presumably due to nonsense mediated decay [43]. This was reflected by the presence of negligible levels of protein in isolated peri-ovulatory oocytes from Mll2Gdf9 cKO females (Figure 1F). Next, by repeated breeding to males of known fertility, we found that Mll2Gdf9 cKO females were sterile (Figure 1G). To begin to understand the basis of the infertility displayed by Mll2Gdf9 cKO mice, we analyzed serum hormone levels. Eight-week-old Mll2Gdf9 cKO females displayed increased serum gonadotropin hormones (FSH: WT, 4.24±0.43 ng/ml; Mll2Gdf9 cKO, 23.81±1.99 ng/ml; and LH: WT, 0.14±0.03 ng/ml; Mll2Gdf9 cKO, 0.87±0.17 ng/ml; n=5; p<0.05) and decreased serum estradiol (WT, 21.66±3.23 ng/ml; Mll2Gdf9 cKO, 12.24±1.0 ng/ml; n=5; p=0.06), suggesting abnormal folliculogenesis.

To investigate further this potential defect, histological sections of ovaries from Mll2Gdf9 cKO females of various ages were evaluated and follicle counts were performed. Based on morphology [44], follicles were grouped into three categories: dormant (primordial), growing (primary, secondary, preantral, and antral), or dying (atretic and zona pellicula remnants). Two-week-old Mll2Gdf9 cKO ovaries displayed a higher number of primary growing follicles (Figure 2A and 2B, upper panels), suggesting increased follicular recruitment. Three-week-old Mll2Gdf9 cKO ovaries showed a 50% decrease in dormant follicles and an increase in growing preantral follicles (Figure 2A and 2B, middle panels), whereas 8-week-old Mll2Gdf9 cKO ovaries displayed an 80% reduction in dormant follicles (Figure 2A and 2B, lower panels), demonstrating increased follicle loss. Further, the number of atretic follicles was significantly increased in Mll2Gdf9 cKO females from 3 wk of age onwards (Figure 2D; n=5, ANOVA test, p<0.05). Older (36-wk-old) Mll2Gdf9 cKO ovaries showed few follicles (Figure 2C). Together, the results suggest that increased early follicle recruitment and oocyte loss contributes to infertility of Mll2Gdf9 females.

Mll2Gdf9 cKO Females Fail to Ovulate

By stimulation of pre-pubertal mice with gonadotropin hormones to induce ovulation and resumption of meiosis, we found that Mll2Gdf9 cKO females were essentially anovulatory (Figure 3A; n=4, Student’s t test, p<0.05). Anovulation was confirmed by histological analysis of ovaries from hormonally stimulated females, which showed oocytes trapped in luteinizing structures (Figure 3B). To determine whether resumption of meiosis was also compromised, we cultured isolated peri-ovulatory oocytes and found that the majority of Mll2Gdf9 cKO oocytes progressed to meiosis II, whereas a small but significant fraction remained arrested in meiosis I (Figure 1C and 1D; ANOVA test; p<0.05).
Oocyte MLL2 Is Required for Female Fertility

A) Control vs. MII2^G4°cKO follicle counts:

- **2 wks**:
  - WT: Prl 2.5, Pr 1.8, Sec 0.5, PA 0.3, Antral 0.2
  - MII2^G4°cKO: Prl 1.2, Pr 0.8, Sec 0.2, PA 0.1, Antral 0.05

- **3 wks**:
  - WT: Prl 2.0, Pr 1.5, Sec 0.5, PA 0.3, Antral 0.2
  - MII2^G4°cKO: Prl 1.0, Pr 0.5, Sec 0.1, PA 0.1, Antral 0.05

- **8 wks**:
  - WT: Prl 1.5, Pr 1.0, Sec 0.3, PA 0.2, Antral 0.1
  - MII2^G4°cKO: Prl 0.8, Pr 0.5, Sec 0.05, PA 0.05, Antral 0.01

B) Follicle counts for WT and MII2^G4°cKO:

- 2 wk-old:
  - WT: Prl 7, Pr 4, Sec 2, PA 1, Antral 0
  - MII2^G4°cKO: Prl 1, Pr 0.5, Sec 0.1, PA 0.1, Antral 0.05

- 3 wk-old:
  - WT: Prl 6, Pr 4, Sec 2, PA 1, Antral 0
  - MII2^G4°cKO: Prl 2, Pr 1, Sec 0.5, PA 0.5, Antral 0.1

- 8 wk-old:
  - WT: Prl 5, Pr 3, Sec 1, PA 0.5, Antral 0.1
  - MII2^G4°cKO: Prl 2, Pr 1, Sec 0.5, PA 0.5, Antral 0.1

C) 36 wk-old ovaries:

- WT: Prl 4, Pr 3, Sec 2, PA 1, Antral 0
- MII2^G4°cKO: Prl 2, Pr 1, Sec 0.5, PA 0.5, Antral 0.1

D) Number of atretic follicles:

- 2 wks: WT 0.5, MII2^G4°cKO 0.8
- 3 wks: WT 0.3, MII2^G4°cKO 0.5
- 8 wks: WT 0.1, MII2^G4°cKO 0.3
but not H3K4me1, suggesting that a histone methyltransferase other than MLL2 is responsible for this methylation mark. The results also indicate that transcriptional repression in peri-ovulatory oocytes is defective in the absence of MLL2. It is unclear whether this phenomenon is a consequence of altered H3K4 methylation, defects in granulosa cell-oocyte communication, or other factors. However, the increased H4K12 acetylation likely reflects the lack of transcriptional silencing observed in Mll2Gdf9 cKO oocytes.

Abnormal Expression Pro-Apoptotic Genes and Iap Elements in Mll2Gdf9 cKO Oocytes

Elevated transcription in Mll2Gdf9 cKO peri-ovulatory oocytes prompted further investigation of potential gene expression changes. A survey of selected genes involved in oocyte function [53–59] and early embryogenesis [60–63] by QPCR analysis of peri-ovulatory oocytes revealed no changes in the majority of genes investigated (Table 1). An exception was the B-type cyclin E3 ubiquitin ligase cyclin B1 interacting protein 1 (Ccnb1ip, also

Figure 2. Mll2Gdf9 cKO females show increased ovarian follicular recruitment and loss. (A) PAS-stained ovaries from 2-, 3-, and 8-wk-old mice; the number of follicles was greatly reduced in ovaries from 8-wk-old Mll2Gdf9 cKO mice. (B) Follicle counts from 2-, 3-, and 8-wk-old mice. At 2 wk, Mll2Gdf9 cKO ovaries showed significantly increased (ANOVA test, *p<0.05) primary follicles (Pr), whereas 3-wk-old Mll2Gdf9 cKO ovaries showed significantly reduced primordial follicles (Prl) and increased preantral follicles (PA) (ANOVA test, *p<0.05). Mll2Gdf9 cKO ovaries from 8-wk-old mice showed further reduction in Prl follicles (ANOVA test, *p<0.05). Means of follicle counts corrected by surface area ± S.E. are shown (ovarian sections from five individual females were used; n = 5). Abbreviations: Prl, primordial; Pr, primary; Sec, secondary; A, antral; CL, corpora lutea. (C) Mll2Gdf9 cKO ovaries from 36-wk-old mice stained with hematoxylin-eosin showed very few follicles (boxed area). (D) From 3 wk onwards, the number of atretic follicles was significantly higher in Mll2Gdf9 cKO ovaries, compared to controls. Means of follicle counts corrected by surface area ± S.E. are shown (ovarian sections from five individual females were used; n = 5). ANOVA test, *p<0.05.

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Figure 3. Mll2Gdf9 cKO females show impaired ovulation. (A) Ovulation rates were significantly reduced in 3-wk-old superovulated Mll2Gdf9 cKO females (Student’s t test, **p<0.01). Means ± S.E. are shown (five individual females were used; n = 5). (B) PAS-stained ovaries from superovulated 3-wk-old Mll2Gdf9 cKO mice showed trapped oocytes (yellow arrowheads), which correlated with decreased ovulation rates in this mouse line. (C) In vitro oocyte maturation studies; representative single plane confocal laser microscopy micrographs of peri-ovulatory oocytes cultured for 16 h to allow resumption of meiosis; arrows show lagging chromosomes. Magnification: 800×. Abbreviations: MI, meiosis I; MII, meiosis II; PB, polar body. (D) Percentages of meiosis I (MI), meiosis II (MII), or abnormal oocytes after 16 h in culture (ANOVA test, *p<0.05). Means ± S.E. of four independent experiments are shown; a total of 120 oocytes per genotype were analyzed.

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known as Hei10, which is important for both meiosis and embryogenesis [64]. Ccnb1ip/Hei10 expression was significantly decreased in Mll2Gdf9 cKO oocytes (p<0.05; Table 1). Interestingly, expression of the histone 4 acetylase Kat5/Tip60 (Lysine acetyltransferase 5), which has been previously reported to be present in oocytes, was significantly increased in Mll2Gdf9 cKO oocytes (Table 1, p<0.05) [65] and may therefore contribute to the observed increase in H4K12 acetylation. QPCR analysis also showed that Mll2Gdf9 cKO oocytes overexpressed several apoptosis-associated genes (Table 1), including p53 (transformation related protein 53; TRP53) and Sed7 (SET domain lysine methyltransferase 7, also known as Set7/9) (Table 1 and Figure S2A), which has been previously reported to be present in oocytes, was significantly increased in Mll2Gdf9 cKO oocytes (Table 1, p<0.05) [65] and may therefore contribute to the observed increase in H4K12 acetylation.

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Finally, because of the widespread lack of transcriptional repression in Mll2Gdf9 cKO oocytes, we looked at the expression of retrotransposon interspersed repeats by QPCR analysis. We found that intracisternal A particle (Iap) elements were abnormally transcribed in Mll2Gdf9 cKO peri-ovulatory oocytes, whereas LINE-1 and SINE-1 elements were not (Figure S3A). Increased Iap expression in Mll2Gdf9 cKO oocytes correlated with hypomethylation of Iap DNA loci, as evaluated by methylation specific primers (Figure S3B) and bisulphite sequencing (Figure S3C). However, Mll2Gdf9 cKO oocytes showed a strong signal for 5-

Figure 4. Mll2Gdf9 cKO oocytes fail to establish transcriptional repression. (A) Confocal laser microscopy analysis of peri-ovulatory oocytes stained with propidium iodide to visualize DNA (upper panel); oocytes were scored as displaying a surrounded nucleolus (SN) or a non-surrounded nucleolus (NSN) configuration (lower panel). Arrows show the nucleolus. No significant differences were observed between controls and Mll2Gdf9 cKO oocytes (ANOVA test, *p=0.08). Means ± S.E. are shown; a total of 170 oocytes from five individual females were analyzed in three independent experiments. Magnification: 800×. (B) Run-on and confocal microscopy analyses of Br-UTP labeled nascent RNA in peri-ovulatory oocytes. Upper panel: Representative single plane confocal micrographs are shown as merge and split channels (Br-UTP, green staining; and DNA: propidium iodide, red staining). Lower panel: oocytes were scored as positive or negative for Br-UTP staining; note that Mll2Gdf9 cKO oocytes were transcriptionally active (ANOVA test, *p<0.05). Transcription was RNA Pol II dependent as it was abrogated by α-amanitin (αA) (magnification: 800×). Means ± S.E. are shown; a total of 90 oocytes from four individual females were evaluated in three independent experiments.

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Oocyte MLL2 Is Required for Female Fertility

A

B

C

D

E

F

G

H

I

WT MII2<sup>gdf6</sup> cKO

H3K4me1

H3K4me2

H3K4me3

Histones

H3K4 methylation

WT MII2<sup>gdf6</sup> cKO

H3K9me2

H3K27me3

H4K20me1

H3 (Ac)

Histones

H4K12 acetylation
methyl-cytosine staining, indicating that loss of CpG methylation was not a widespread phenomenon (Figure S3D). Because retrotransposons drive the expression of multiple host genes in female gametec[72], Iap up-regulation could contribute to loss of Mll2cKO cKO oocytes. Absence of Utyl1 (ubiquitin-like, containing PHD and RING fingers) also known Npy95 in cell lines and mouse embryos has been associated with increased transcription of interspersed repeats due to DNA hypomethylation [73]. Consequently, we examined Utyl1 expression levels and found a 2-fold decrease (Table 1), suggesting a role in Iap deregulation. Decreased Utyl1 in Mll2cKO cKO oocytes could also contribute to Cdkn1a deregulation [74]. Together, the results suggest that stabilization of p53 and abnormal expression of its downstream targets as well as deregulated expression of retrotransposon elements may trigger apoptosis in Mll2cKO cKO oocytes, leading to oocyte death and, ultimately, premature follicle loss and sterility.

Deletion of Mll2 Using Zp3-Cre

To further substantiate the phenotype observed in the Mll2cKO cKO mouse model, we employed conditional mutagenesis using the Zp3-Cre allele (Figure 6A). Peri-ovulatory oocytes from Mll2−/−, Zp3Cre−− females (herein called Mll22cKO cKO) show a significant decrease in Mll2 expression as evaluated by QPCR analysis (Figure 6B, Student’s t test; p<0.05; n=3). Similar to Mll2cKO cKO mice, Mll2−/− cKO females showed a significant increase in FSH levels (Figure 6C) and were infertile (Figure 6D). However, Mll2−/− cKO females showed a milder rate of follicle loss than that of Mll22cKO cKO mice, as evidenced by the lack of significant changes in the number of atretic follicles at 3 wk of age (Figure 6E and 6F) and the presence of follicles at 36 wk of age (Figure 6E). In contrast to Mll22cKO cKO females, which were anovulatory, Mll2−/− cKO mice displayed an ovulation rate of 20%–25% compared to controls (Figure 6G). Consistent with decreased ovulation rates, Mll2−/− cKO ovaries showed a number of oocytes trapped in luteinizing structures (Figure 6H). Interestingly, oocytes ovulated by Mll2−/− cKO females were capable of fertilization_in vitro, and the majority of embryos generated from matings of Mll2−/− cKO females and WT males became developmentally arrested between the 1-cell and 2-cell stages (Figure 6I). Confocal microscopy analysis of Mll2−/− cKO oocytes showed an increase in the levels of H3K4me1 (Figure 7A) and a reduction in the levels of H3K4me3 (Figure 7B) comparable to that observed in Mll22cKO cKO oocytes (Figure 3A–C). Western blot analysis confirmed the decrease in bulk H3K4me3 in Mll2−/− cKO oocytes (Figure 7C). These results demonstrate that
Table 1. Real time PCR analysis of molecular changes in Mll2<sup>tm2</sup>cKO oocytes.

| Gene       | Control | cKO       |
|------------|---------|-----------|
| Mll3       | 1.02±0.26 | 0.70±0.22 |
| Setd1a     | 1.03±0.28 | 1.11±0.17 |
| Setd8      | 1.03±0.16 | 1.07±0.15 |
| Smyd3      | 1.06±0.05 | 0.87±0.18 |
| Wdr5       | 1.01±0.10 | 1.07±0.30 |
| Ash2l      | 1.01±0.11 | 1.08±0.30 |
| Rbbp5      | 0.92±0.04 | 2.27±0.34* |
| Uhrf1      | 0.94±0.07 | 0.43±0.04* |
| Tip60/Kat5 | 1.01±0.07 | 8.53±2.03* |

Oocyte function and meiosis

| Gene       | Control | cKO       |
|------------|---------|-----------|
| Gdf9       | 1.00±0.12 | 1.06±0.18 |
| Bmp15      | 1.69±0.36 | 1.43±0.72 |
| Pten       | 1.00±0.10 | 1.53±0.35 |
| Kit        | 0.87±0.11 | 0.76±0.06 |
| Taf2       | 0.99±0.10 | 0.94±0.07 |
| Pde3a      | 0.99±0.11 | 1.29±0.51 |
| Ccnb1ip1   | 1.11±0.12 | 0.04±0.01* |
| Apoptosis/p53 |        |           |
| Bcl2       | 1.02±0.17 | 0.91±0.09 |
| Casp6      | 1.00±0.08 | 1.46±0.04* |
| Bax        | 1.06±0.17 | 1.92±0.18* |
| Cdk1a/p21  | 1.06±0.06 | 2.07±0.37* |
| Fos        | 1.37±0.43 | 6.29±0.57* |
| Bbc3/Puma  | 1.23±0.23 | 1.91±0.34 |
| Setd7      | 1.05±0.07 | 1.87±0.27* |
| p53        | 1.15±0.10 | 2.12±0.20* |

Development

| Gene       | Control | cKO       |
|------------|---------|-----------|
| Nalp5      | 1.10±0.47 | 1.03±0.50 |
| Dopp3a     | 1.07±0.22 | 0.98±0.23 |
| Smarca4    | 1.07±0.18 | 0.85±0.31 |

Mll2<sup>tm2/tm2</sup> Female Mice Show Reduced Fertility

We generated an FFP-Mll2 fusion protein by gene targeting in ES cells and name the allele targeted mutation 2 (Mll2<sup>tm2</sup>; Figure S4A). Homozygous mice carrying the Mll2<sup>tm2</sup> allele (Mll2<sup>tm2/tm2</sup>) were viable (Figure S4B and S4C); however, they showed no detectable yellow fluorescent signal in any tissue (unpublished data). To determine whether FFP-Mll2 was expressed, we analyzed Mll2<sup>tm2/tm2</sup> ovaries by real-time PCR and Western blot and found a significant decrease in both mRNA (Figure S4D) and protein (Figure S4E) levels, indicating that the mice carrying this allele were hypomorphs for MLL2. We next examined the fertility of Mll2<sup>tm2/tm2</sup> females by mating them to males of known fertility. From the first month of mating, Mll2<sup>tm2/tm2</sup> females showed a 50% reduction in the number of pups per litter compared to WT or Mll2<sup>gnd2/+</sup> controls, and showed impaired cumulative fertility with age (Figure S4F). Notably, the fertility of Mll2<sup>gnd2/+</sup> females was also cumulatively reduced (Figure S4F).

Embryos from Mll2<sup>tm2/tm2</sup> Females Show Decreased In Vitro Developmental Potential

Ovaries from Mll2<sup>tm2/tm2</sup> females appeared normal until 4 mo of age (Figure S4G). Unlike the oocyte-specific cKO mouse lines, ovulation rates of hormonally stimulated pre-pubertal Mll2<sup>gnd2/+.tm2</sup> and control mice were comparable (Mll2<sup>gnd2/+.tm2</sup>: 42±3.0; Mll2<sup>gnd2/+</sup>: 34.0±5.9; n = 4). Furthermore, fertilization rates of young control and Mll2<sup>gnd2/+.tm2</sup> females were also comparable (WT: 30.0±2.7; Mll2<sup>gnd2/+.tm2</sup>: 28.0±2.7; Mll2<sup>gnd2/+.tm2</sup>: 29.4±3.9; n = 12). These findings suggested that subfertility in Mll2<sup>tm2/tm2</sup> females was due to embryonic defects. In embryo cultures, most embryos from Mll2<sup>tm2/tm2</sup> intercrosses (75%) arrested between the 1-cell and 4-cell stages whereas >70% of WT embryos developed to the blastocyst stage regardless of the male genotype (Figure S4H). This phenotype was very similar to that of embryos obtained from crossing Mll2<sup>tm2</sup> cKO females to WT males. Additionally, a higher percentage of fragmentation (22%), which indicates decreased survival, was observed in Mll2<sup>tm2/tm2</sup> embryos compared to controls (5%). The severity of this outcome was slightly relieved when Mll2<sup>tm2/tm2</sup> females were crossed to WT males (Figure S4I), indicating that expression of Mll2 from the paternal allele could partially rescue the defect. Development was also impaired when Mll2<sup>gnd2/+</sup> females were mated to Mll2<sup>gnd2/+.tm2</sup> or even WT males (Figure S4H and S4I).

Mll2<sup>tm2/tm2</sup> Embryos Show Both Reduced ZGA and Bulk H3K4 Methylation

Because the majority of the Mll2<sup>tm2/tm2</sup> embryos arrested before the 8-cell stage, we evaluated their ability to undergo ZGA using synthesis of the Transcription-Requiring Complex [75] as a marker. Embryos from Mll2<sup>tm2/tm2</sup> intercrosses showed a 30% reduction in H3K4me2 levels compared to controls (Figure S5A and S5B; n = 5, p<0.05). Therefore, defective ZGA likely contributes to the developmental block observed in Mll2<sup>tm2/tm2</sup> embryos.

To investigate the potential link between MLL2 activity and ZGA, we analyzed the status of H3K4 methylation in Mll2<sup>gnd2/+</sup> embryos. Immunofluorescence and confocal microscopy (Figure S6) showed decreased levels of H3K4me2 and H3K4me3 in female pronuclei of 1-cell embryos from Mll2<sup>gnd2/+</sup> intercrosses (Figure S6B and S6C), whereas control embryos showed the expected asymmetry for H3K4 methylation between the female and male pronuclei (Figure S6B and S6C) [31]. Western blot analysis of chromatin fractions and chemoluminescence quantification confirmed a significant reduction in H3K4me3 levels in Mll2<sup>gnd2/+</sup> zygotes (Figure S6, left and right panels). The results from the hypomorphic allele are in agreement with and extend the results from conditional mutagenesis of Mll2. Near-ubiquitous mutagenesis of Mll2 in female adults caused infertility (Figure S1). Removal of Mll2 from oocytes but not from granulosa cells also caused infertility. Gdf9<sup>-</sup>-cre-mediated deletion of Mll2 caused a more severe phenotype than Zfp3<sup>-</sup>-cre-mediated deletion, consistent with the earlier expression of Gdf9 in oocytes. The hypomorphic allele of Mll2 presented the mildest phenotype, consistent with a reduction, but not abolishment of MLL2 protein levels (Figure S4E).

Discussion

In this article, we identify MLL2 as one of the major factors controlling bulk H3K4 methylation during oocyte growth and pre-implantation development. Our results indicate that MLL2 is...
Oocyte MLL2 Is Required for Female Fertility

A. Breeding scheme

\[ \text{MLL2}^{F/f} \times Zp3-Cre^* \]
\[ \downarrow \]
\[ \text{MLL2}^{F/f}, Zp3-Cre^* \times \text{MLL2}^{ff} \]
\[ \downarrow \]
\[ \text{MLL2}^{F/f} \text{ (control)}; \]
\[ \text{MLL2}^{F/f}, Zp3-Cre^* \text{ (MLL2}^{Zp3} \text{cKO)} \]

B. MLL2 mRNA

RQ

\[ \begin{array}{c|c|c}
\text{WT} & \text{MLL2}^{Zp3} \text{cKO} \\
0.4 & * \\
\end{array} \]

C. FSH levels

FSH (ng/ml)

\[ \begin{array}{c|c|c}
\text{control} & \text{MLL2}^{Zp3} \text{cKO} \\
2 & * \\
\end{array} \]

D. Fertility studies

Total number of pups

\[ \begin{array}{c|c|c}
\text{Time (months)} & \text{Control} & \text{MLL2}^{Zp3} \text{cKO} \\
0 & 50 & 50 \\
1 & 100 & 100 \\
2 & 150 & 150 \\
3 & 200 & 200 \\
4 & 250 & 250 \\
5 & 300 & 300 \\
6 & 350 & 350 \\
\end{array} \]

E. Histological images

Control

MLL2^{Zp3} cKO

3-wks

36-wks

65X

52X

F. Follicle counts

\[ \begin{array}{c|c|c|c|c|c|c|c|c|c}
& \text{Prl} & \text{Pr} & \text{Sec} & \text{PA} & \text{A} & \text{AT} & \text{Prl} & \text{Pr} & \text{Sec} & \text{PA} & \text{A} & \text{CL} & \text{AT} \\
3 \text{ wk-old} & & & & & & & & & & & & \\
& & & & & & & & & & & & \\
& * & * & * & & & & * & * & * & & & \\
8 \text{ wk-old} & & & & & & & & & & & & \\
& & & & & & & & & & & & \\
& & & & & & & & * & * & * & & & \\
\end{array} \]

G. Ovulation rates

\[ \begin{array}{c|c|c|c|c}
& \text{WT} & \text{MLL2}^{Zp3} \text{cKO} \\
\# ovulated oocytes & & \\
& 50 & 50 \\
\# ovulated oocytes & & \\
& 45 & 45 \\
\# ovulated oocytes & & \\
& 40 & 40 \\
\# ovulated oocytes & & \\
& 35 & 35 \\
\# ovulated oocytes & & \\
& 30 & 30 \\
\# ovulated oocytes & & \\
& 25 & 25 \\
\# ovulated oocytes & & \\
& 20 & 20 \\
\# ovulated oocytes & & \\
& 15 & 15 \\
\# ovulated oocytes & & \\
& 10 & 10 \\
\# ovulated oocytes & & \\
& 5 & 5 \\
\# ovulated oocytes & & \\
& 0 & 0 \\
\end{array} \]

H. MLL2^{Zp3} cKO superovulated ovaries

I. In vitro embryo development

% embryos

\[ \begin{array}{c|c|c|c|c|c}
\text{embryo stage} & \text{WT} & \text{MLL2}^{Zp3} \text{cKO} \\
1c & & & & & \\
2c & * & * & & & \\
4c & & & & & \\
8c & & & & & \\
Frag & & & & & \\
\end{array} \]

* indicates significant difference.
autonomously required in the oocyte for normal oogenesis and pre-implantation development. Oocyte-specific cKO females showed severe defects in fertility, which were also observed in both conditionally mutated and hypomorphic adult females, whereas granulosa cell-specific cKO females showed almost normal fertility. The results suggest differential regulation in H3K4 methylation in gametes and somatic cells. Unlike cKO oocytes (Table 1), $\text{Mll2}_{\text{Amhr2}}$ cKO granulosa cells showed an increase in $\text{Mll3}$, which could compensate, at least partially, for the loss of $\text{Mll2}$.

Figure 6. Oocyte-specific deletion of $\text{Mll2}$ mediated by the $\text{Zp3-Cre}$ allele. (A) Breeding scheme used to generate $\text{Mll2}^{\text{Zp3-cKO}}$ mice. (B) Zp3-cKO oocytes show a significant reduction in Mll2 mRNA levels (Student’s t test, * $p<0.05$). Results are shown as means ± S.E. relative to WT (RQ) (three oocyte pools were used; $n=3$) and Gapdh was used as endogenous control. (C) FSH levels were significantly higher in serum samples from 8-wk-old $\text{Mll2}^{\text{Zp3-cKO}}$ females compared to controls (Student’s t test, * $p<0.05$; $n=10$). (D) Fertility studies shown as cumulative number of pups over a 6-mo period. $\text{Mll2}^{\text{Zp3-cKO}}$ females were infertile. (G) Ovulation rates were significantly reduced in $\text{Mll2}^{\text{Zp3-cKO}}$ females compared to controls (WT) (Student’s t test; * $p<0.05$; $n=5$). (E) PAS-stained ovaries from 3- and 36-wk-old mice. Original magnification: 25 × and 50 ×; note that different from $\text{Mll2}_{\text{Gdf9}}$ cKO mice, $\text{Mll2}^{\text{Zp3-cKO}}$ ovaries still contain follicles at 36 wk of age. (F) Follicle counts in $\text{Mll2}^{\text{Zp3-cKO}}$ cKO ovaries. $\text{Mll2}^{\text{Zp3-cKO}}$ ovaries had a significantly lower number of primordial follicles and increased numbers of secondary and multilayer preantral follicles 3 wk of age (left panel). By 8 wk of age (right panel), the number of primordial and multilayer preantral follicles is significantly reduced and the number of atretic follicles is increased in $\text{Mll2}^{\text{Zp3-cKO}}$ ovaries. (ANOVA test; * $p<0.05$; ovarian sections from five females were used in the analysis; $n=5$). Abbreviations: Prl, primordial; Pr, primary; Sec, secondary; PA, preantral; A, antral; CL, corpora lutea; Atr, atretic. (I) Cultures of embryos from $\text{Mll2}^{\text{Zp3-cKO}}$ females crossed to WT males showed a developmental arrest between the 1-cell and the 4-cell stages. Results are presented as the % embryos (average) ± standard error from five independent experiments. ANOVA test; * $p<0.05$. A total of 95 embryos from six females per genotype were used in three independent experiments. Abbreviations: C, cell; Frag, fragmented; WT, control wild type.

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Figure 7. $\text{Mll2}^{\text{Zp3-cKO}}$ oocytes show decreased H3K4 tri-methylation. (A–B) Confocal microscopy micrographs showing H3K4 methylation levels in peri-ovulatory oocytes. (A) An increase in H3K4me1 is apparent in $\text{Mll2}^{\text{Zp3-cKO}}$ oocytes (lower panel) compared to control (upper panel). In contrast, a decrease in H3K4me3 (B) levels was observed in $\text{Mll2}^{\text{Zp3-cKO}}$ oocytes (lower panel) compared to controls (upper panel). Magnification: 800 ×. Representative single plane micrographs are shown as merge and split channels of histone tail modifications (green) and DNA (red). (C) Representative micrographs of Western blots showing H3K4me3 levels in chromatin fractions from $\text{Mll2}^{\text{Zp3-cKO}}$ peri-ovulatory oocytes. A decrease in global H3K4me3 is apparent in $\text{Mll2}^{\text{Zp3-cKO}}$ oocytes (Student’s t test, * $p<0.05$). Three pools of 100 oocytes each from 3–4 females per genotype were used in three independent experiments. Total histones were used as internal loading controls.

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Our studies demonstrate that MLL2 is required during the transcriptionally active period of oocyte growth for the establishment and/or maintenance of bulk H3K4 tri-methylation (Figure 9). A previous study showed that H3K4 methylation increases from immature (meiotically incompetent) oocytes to peri-ovulatory stage (meiotically competent) oocytes [22]. That study also showed that the expression of the H3K4 methyltransferases Mll1 and Smyd2 decreased towards the peri-ovulatory stage, suggesting that a different methyltransferase is responsible for H3K4 methylation in peri-ovulatory oocytes. Here, we show that Mll2 expression increases towards the peri-ovulatory stage and that its loss in oocytes leads to decreased H3K4 methylation. Our results are consistent with the idea that MLL2 is responsible for the increase in H3K4 methylation in peri-ovulatory oocytes observed by Kageyama and collaborators. In addition, the results suggest that although MLL2 is required throughout oogenesis, MLL2 might be particularly important as oocytes develop meiotic competence.

Changes in bulk H3K4me3 methylation (Figures 5, 7, and 9) observed in oocytes were unexpected because loss of Mll2 in ES cells or adult mice did not affect bulk H3K4me3 [39,76]. Our findings demonstrate that MLL2 is responsible for the majority of H3K4me3 in oocytes and that a different enzyme(s) is responsible for H3K4me1. Mono-methylation by dedicated monomethylases that co-operate with dedicated di-/tri-methylases has been observed for other histone tail lysines but not for H3K4 [35]. Until now, there has been no evidence that a similar mechanism may operate on mammalian H3K4 methylation [77], thereby differing from the paradigm H3K4 methyltransferase, yeast Set1C, which mediates mono-, di-, and tri-methylation under the control of its various subunits [36,78,79].

Oocytes lacking Mll2 displayed a variety of defects including lack of global transcriptional silencing at the peri-ovulatory stage and abnormal expression of Utrophin, Canblip1, Iap, and pro-apoptotic factors (Table 1 and Figures S2, S3, and 9). The failure to undergo transcriptional silencing is paradoxical because MLL2, like other trithorax-Group factors, acts to maintain expression of target genes [39]. A possible explanation for this paradox is that MLL2 is required for expression of key factors that mediate global transcriptional silencing, and therefore, this defect is likely to be an indirect consequence of Mll2 loss. Previous studies have shown that granulosa cell-oocyte communication is essential for transcriptional repression in oocytes [12]. Thus, it is possible that altered communication between the gamete and somatic cell compartments in Mll2KO cKO ovaries, as evidenced by the ovoluotary defect, may contribute to the lack of transcriptional repression. A previous study showed that global transcriptional silencing occurred without the large chromatin rearrangement into the SN configuration [1]. Here we extend this observation by showing the converse; acquisition of the SN configuration in Mll2-deficient oocytes occurred without global transcriptional silencing, confirming that these two events are independent.

It is likely that the observed overexpression of Iap elements and apoptotic factors in Mll2-deficient oocytes is an indirect consequence of Mll2 loss. In contrast, the decrease in the expression of Utrophin and Canblip1 may reflect direct regulation of these genes by MLL2, since both genes are expressed from CpG islands, as are all MLL2 targets described to date [39].

Results in the hypomorph and Mll2<sup>−/−</sup> cKO models demonstrated a developmental arrest between the 1- and 4-cell stages, when ZGA takes place in mouse embryos. In addition, Mll2 hypomorph zygotes displayed impaired ZGA (Figure 9). A possible explanation for this phenotype is that unknown maternal factors required for ZGA are lacking in Mll2-deficient oocytes. Another possibility is that the establishment of H3K4 di- and tri-methylation in the oocyte by MLL2 contributes to establish a poised starting point for the activation of the embryonic genome after fertilization. Finally, it is also possible that MLL2 itself might be required to activate transcription in the zygote. In this context, we note that MLL2 appears to be continuously required after fertilization during at least the first three cell divisions (Figure 9). This conclusion is supported by the fact that MLL2 is expressed in pre-implantation embryos from the 2-cell stage onwards and that embryos derived from Mll2<sup>−/−</sup> cKO and Mll2<sup>−/−</sup> <sup>gdm2</sup> females undergo developmental arrest. Recently, an unidentified MLL2 protein has been implicated as part of a BRG-1 containing complex required for ZGA [60]. Our results from the hypomorph model suggest that MLL2 could be this protein.

Finally, recent studies have shown that H3K4 histone methyltransferases, including MLL2, may also play a role in nuclear receptor activation and hormone signaling, at least in human cells [80]. Thus, defective signaling pathways could also contribute to the phenotype observed in the various Mll2 cKO mouse lines. Undoubtedly, more studies are needed to dissect the potential role of MLL2 in these pathways.

Together with previous studies [37], our findings suggest that during development there is a switch from an MLL2-dependent to MLL2-independent state. In the oocyte, MLL2 is the major tri-H3K4 methyltransferase, and maternal MLL2 is required for the first embryonic divisions. Previous studies showed that Mll2 KO mice develop up to E9.5 [37], suggesting that an MLL2-independent state is established after ZGA. We hypothesize that a cell-type specific requirement for MLL2 may be established later in development during gastrulation. This implies a cell-type specific switch in epigenetic roles, which points to a new dimension of complexity in epigenetic regulation.

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Oocyte MLL2 Is Required for Female Fertility

Epigenetic changes

Primordial
Primary
Preantral
Antral
Pre-ovulatory
Mll oocytes
1 cell
2 cell
Blastocyst (E 3.5)

DNA methylation
Histone tail modifications

MLL2 deficiency
Lap demethylation
Increased pro-apoptotic gene expression

H3K4me2/3 loss
But not H3K4me1
High H4K12ac

MLL2 requirement
SN
TR

SN but not TR
No ovulation
Follicle loss

Deficient ZGA
Embryo lethality (cKO, hypomorph)
Our results indicate that MLL2 is required during post-natal oogenesis and the 2–3 embryonic cell divisions, thereby identifying MLL2 as one of the key players in the epigenetic reprogramming required for female fertility in the mouse. Given the recent interest in epigenetics raised by the reprogramming of somatic cells to a pluripotent state equivalent to the epiblast, termed iPSCs (induced pluripotent stem cells), it will be interesting to determine whether MLL2 is also required for the generation of iPSCs. If this holds true, MLL2 dependency could be used as a criterion to determine whether reprogramming to iPSC cells recapitulates endogenous mechanisms or is largely synthetic short-circuiting.

Materials and Methods

Generation of Mouse Lines

Conditional KO mice were generated by crossing Rosa26-CreERT2 [81] Gdf9-Cre [40], zona pellucida 3 (Zp3)-Cre [41], or anti-Mullerian hormone receptor 2 (Andr2)-Cre [42] mice and Mll2+/− and Mll2−/− mice [37] as explained in Text S1. The hypomorph line Mll2Hypom was generated inadvertently when a yellow fluorescence protein (YFP) tag was added in frame with the first ATG of Mll2 located in exon 1 (Figure 1A, Text S1). All mouse lines were maintained in the 129/C57BL/6 hybrid background. Genotyping from tail DNA was performed as described [37,40,42]. The decrease in Mll2 levels was confirmed by quantitative PCR (QPCR) analysis using primers described in Table S1. Mice were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals and under German license nr. 24-9168.11-1-2004-26.

Fertility Studies, Serum Hormone Levels, Histological Analysis, Cell Collection, and Culture

Fertility studies, serum hormone measurements, and histological analysis were performed as previously described [82]. More details are provided in Text S1. 21-d-old females were injected with 5 IU of pregnant mare serum gonadotropin (PMSG; Calbiochem, EMD, Gibbstown, NJ), and granulosa cells or peri-ovulatory oocytes were collected 47 h later [82]. Superoxovulation and oocyte rates in 21-d-old females were carried out as described [82]. In vitro oocyte maturation and embryo cultures are detailed in Text S1.

RT-PCR and Quantitative Real Time PCR (QPCR) Analyses

RNA from oocytes or granulosa cells was isolated and subjected to QPCR analysis as described in Text S1. Primer sets are described in [69] and Table S1. Primer amplification efficiency and transcript levels were calculated as previously described [82]. Gapdh was used as endogenous control. The relative amount of target gene expression for each sample was presented as the mean ± SEM.

Chromatin Isolation and Western Blot Analysis

Chromatin was isolated from oocytes or granulosa cells without nuclease treatment [83] and equal amounts of protein or equal numbers of oocytes were fractionated in 4%–12% SDS-PAGE Bis-Tris gels (Invitrogen, Carlsbad, CA). Total histones were determined by SYPRO Ruby protein blot stain (Molecular Probes, Invitrogen). Proteins were detected with Super-signal West Pico-detection system (Pierce/Thermo Fisher Scientific, Pittsburgh, PA), and levels were quantified by using the Image J software (NIH). Results are presented as a ratio of modified histone to total histones or protein to tubulin and represent the average of three pools collected in three experiments.

Immunofluorescence and Confocal Laser-Scanning Microscopy

Peri-ovulatory oocytes were fixed and stained as previously described [84]. Samples were mounted in well-slides (Fisher Scientific) using Vectashield containing propidium iodine (Vector Laboratories, Burlingame, CA). Fluorescence was detected on a laser-scanner confocal microscope (Carl Zeiss, Thornwood, NY). Images were collected at 40× magnification with zoom level equal to 2. For each experimental series, oocytes from all groups were processed under the same conditions and images were captured using the same microscope settings.

Metabolic Labeling, TRC Complex Detection, Run-On Assays, and DNA Methylation Assays

Metabolic labeling of 2-cell embryos, protein extraction, and TRC detection were performed as previously reported [85,86]. Transcriptional activity was determined in run-on studies after 5-bromo uridine 5’-triphosphate (BrUTP, Sigma) incorporation as previously described [12,46]. A detailed explanation is presented in Text S1. Global DNA methylation was assessed by immunostaining against 5-methylcytosine, and Iap DNA methylation was determined by bisulphite genomic sequencing [73] or bisulphite conversion followed by PCR analysis using methylation-specific primers, which were designed using MethPrimer.

Data Analysis

Statistical significance was determined by Student’s t test or one-way analysis of variance (ANOVA) and test for multiple comparison analysis Newman-Keuls’s (SNK’s); p values <0.05 were considered to be significant.

Supporting Information

Figure S1 Loss of Mll2 in adult females using tamoxifen-induced Cre recombination leads to infertility. Mll2−/−; Rosa26CreERT2/− (Mll2−, conditional KO, grey bars) or Mll2−/−; Rosa26CreERT2/+/− (control, black bars) females
(upper panel) were mated to WT males. Initially ten 2-mo-old adults of each genotype were mated to establish fertility before induction, then they were treated with tamoxifen as described [30] and tested for fertility. The WT males (one male for each pair of tamoxifen treated females) were exchanged every week. Data show the number of successful litters. Note that tamoxifen treatment provokes infertility in females regardless of genotype. After treatment was discontinued, control animals fully recover fertility, whereas Mll2 conditional KO females remain infertile.

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Figure S2 Mll2Gdf9 cKO oocytes show increased expression of the methyltransferase Setd7, p53 stabilization, and expression of p53 downstream pro-apoptotic target genes. (A) QPCR analysis showed a significant increase in the methyltransferase Setd7 in Mll2Gdf9 cKO peri-ovulatory oocytes (Student’s t test, * p<0.05; three pools of oocytes were used in the analysis (n = 3)). Gapdh was used as endogenous control. (B) Representative Western blot analysis showing increased p53 protein levels in Mll2Gdf9 cKO peri-ovulatory oocytes; tubulin was used as loading control. Note that p53 is normally absent in peri-ovulatory stage oocytes. (C) QPCR analysis showed a significant increase in the expression of p53 target genes, which are involved in apoptosis, including Bax, Cldn11, Foxo, and Casp6 in Mll2Gdf9 cKO peri-ovulatory oocytes (Student’s t test, * p<0.05; ** p<0.01; three pools of oocytes were used in the analysis (n = 3)). Gapdh was used as endogenous control.

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Figure S3 Mll2Gdf9 cKO oocytes display abnormal levels of the retrotransposable element Iap. (A) QPCR analysis of retrotransposon mRNA in isolated peri-ovulatory Mll2Gdf9 cKO oocytes showed an increase (Student’s t test, * p<0.05) in Iap (intracisternal A particle) but not in LINE-1 (Long Interspersed Nuclear Element 1, L1) or SINE-1 (Short Interspersed Nuclear Element); means ± S.E. are shown (three pools of 100 oocytes each were used in the analysis; n = 3). (B) Methylcytosine-specific PCR analysis of the Iap promoter showed a significant decrease in CpG DNA methylation in peri-ovulatory Mll2Gdf9 cKO oocytes (Student’s t test, * p<0.05); means ± S.E. are shown (three pools of 100 oocytes each were used in the analysis; n = 3). (C) Hypomethylation of Iap in peri-ovulatory Mll2Gdf9 cKO oocytes was confirmed by bisulfite sequencing. Methylated and unmethylated CpGs are shown as filled or open circles, respectively. (D) 5-methylcytosine (5-Mc-C) staining and confocal microscopy analysis reveal that loss of DNA methylation in peri-ovulatory Mll2Gdf9 cKO oocytes is not a widespread phenomenon. Merge and grayscale split channels (5-Mc-C, FITC; DNA, propidium iodide, red) of single plane confocal sections are shown. Magnification: 800×.

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Figure S4 Generation and characterization of Mll2tm2Jgot/tm2Jgot ((Mll2gtn2/tm2) mice). (A) Schematic representation of targeted mutation 2 Mll2gtn2 (denoted throughout as Mll2tm2). An enhanced yellow fluorescence protein (eYFP) cassette was introduced in the N terminus end of Mll2, followed by a neomycin (neo) cassette flanked by two Frt (FLP recombination target) sites. FLPc (Flip)-mediated recombination resulted in a continuous reading frame from the authentic Mll2 initiating codon, through eYFP and the residual FRT to the second amino acid of Mll2. (B) Mice carrying a single copy of Mll2gtn2 (Mll2gt1/2) were intercrossed to obtain control (Mll2 +/+ and Mll2gtn2/tm2) and experimental Mll2gtn2/tm2 mice. (C) Southern blot analysis of tail genomic DNA: a 3.8 kb band denotes the Mll2gtn2/tm2 allele, whereas a 3.0 kb band denotes the wild type (WT) allele. Mll2gtn2/+ mice are distinguished by the presence of the two bands. (D) Real time PCR analysis showing a small but significant decrease in Mll2 mRNA levels in ovaries from Mll2gtn2/tm2 females (ovaries from three females were used in the analysis (n = 3); Student’s t test, p<0.05). (E) Representative Western blot analysis showing a decrease in Mll2 levels in ovaries from Mll2gtn2/tm2 females; tubulin was used as loading control. (F) Fertility studies shown as cumulative number of pups over a 6-mo period. Females were mated to WT males. The cumulative number of pups produced by Mll2gtn2/tm2 females was lower than that of controls indicating subfertility (n = 10 per genotype). (G) PAS-stained ovaries from 3-wk-old mice. Mll2gtn2/tm2 ovaries show normal follicular development at this age. Original magnification: 50×. (H) Developmental potential of Mll2gtn2/tm2 embryos. Pre-pubertal (21-d-old) females were superovulated and mated to 6-wk-old Mll2gtn2/tm2 males, which showed no defects in fertility at this age (H) or WT males (I). Embryonic development was evaluated in vitro. Means ± S.E. from five independent experiments are shown (approximately 35 embryos per each female, from a total of 8–11 females per genotype, were used in the analysis). (H) When females were mated to Mll2gtn2/tm2 males, a significantly lower percentage of Mll2gtn2/tm2 embryos reached the blastocyst stage; embryos accumulated between 1C and 4C stages. In contrast, the majority of embryos from WT females developed to blastocysts. (I) When females were mated to WT males, few embryos from Mll2gtn2/tm2 females reached the blastocyst stage. Abbreviations: 1C, 1-cell; 2C, 2-cell; M, morula; Bl, blastocyst. ANOVA test; * p<0.05.

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Figure S5 Mll2tm2/tn2 embryos show defects in zygote genome activation (ZGA). (A) Representative autoradiogram showing Transcription Required Complex (TRC) complex levels. Two-cell embryos underwent metabolic labeling to determine TRC synthesis, as a marker of ZGA. Note the reduction in TRC levels in Mll2tm2/tn2 2-cell embryos (Student’s t test, * p<0.05) in Mll2tm2/tn2 2-cell embryo pools (three pools of embryos from six females per genotype were used in this experiment; n = 3); α-amanitin-(α-a)-treated embryos were used as negative controls. (B) Quantification of TRC complex levels. TRC was significantly reduced (Student’s t test, * p<0.05) in Mll2tm2/tn2 2-cell embryo pools (three pools of embryos from six females per genotype were used in this experiment; n = 3).

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Figure S6 Mll2tm2/tn2 embryos show defects in H3K4 bulk methylation. (A-C) Representative micrographs showing confocal microscopy analysis of H3K4me1 (A), H3K4me2 (B), and H3K4me3 (C); a decrease in H3K4 di- and tri-methylation was observed in the female (f) pronucleus of Mll2tm2/tn2 embryos; the male pronucleus (m) is negative for H3K4me2 and H3K4me3, as expected; polar bodies (PB) stain with all antibodies used, as previously reported. Merge and grayscale split channels (H3K3 methylated, FITC; DNA, propidium iodide, red) of single plane confocal sections are shown. (D-E) Western blot analysis of H3K4 methylation in chromatin fractions from zygotes. Three pools of 100 zygotes each from 3–4 females per genotype were used in three independent experiments. Total histones were used as internal loading control. (D) Representative micrographs of Western blots showing H3K4me1 and H3K4me3 levels. (E) Chemoluminescence quantification revealed a significant decrease in global H3K4me3 levels in Mll2tm2/tn2 embryos; samples were normalized against total histones. Student’s t test, * p<0.05.

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Table S1 List of primer sequences.
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Author Contributions
The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: CVAV SG KA AFSS MM MM. Performed the experiments: CVAV SG KA. Analyzed the data: CVAV. Contributed reagents/materials/analysis tools: AFSS MMM. Wrote the paper: CVAV AFSS. Revised the manuscript: CVAV MMM.

Maintenance of mouse colonies/genotyping: RC JEA. Performed studies in the Tamoxifen-Cre line and preliminary studies in Mll2 hypomorph; designed original Mll2 KO and Floxed alleles: SG KA.

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Author Contributions
The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: CVAV SG KA AFSS MM MM. Performed the experiments: CVAV SG KA. Analyzed the data: CVAV. Contributed reagents/materials/analysis tools: AFSS MMM. Wrote the paper: CVAV AFSS. Revised the manuscript: CVAV MMM.

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