Histone Deacetylase 2 (HDAC2) Regulates Chromosome Segregation and Kinetochore Function via H4K16 Deacetylation during Oocyte Maturation in Mouse

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Abstract

Changes in histone deacetylation occur during oocyte development and maturation, but the role of specific histone deacetylases in these processes is poorly defined. We report here that mice harboring Hdac1−/−/Hdac2−/− or Hdac2−/− oocytes are infertile or sub-fertile, respectively. Depleting maternal HDAC2 results in hyperacetylation of H4K16 as determined by immunocytochemistry—normal deacetylation of other lysine residues of histone H3 or H4 is observed—and defective chromosome condensation and segregation during oocyte maturation occurs in a sub-population of oocytes. The resulting increased incidence of aneuploidy likely accounts for the observed sub-fertility of mice harboring Hdac2−/− oocytes. The infertility of mice harboring Hdac1−/−/Hdac2−/− oocytes is attributed to failure of those few eggs that properly mature to metaphase II to initiate DNA replication following fertilization. The increased amount of acetylated H4K16 likely impairs kinetochore function in oocytes lacking HDAC2 because kinetochores in mutant oocytes are less able to form cold-stable microtubule attachments and less CENP-A is located at the centromere. These results implicate HDAC2 as the major HDAC that regulates global histone deacetylation during oocyte development and, furthermore, suggest HDAC2 is largely responsible for the deacetylation of H4K16 during maturation. In addition, the results provide additional support that histone deacetylation that occurs during oocyte maturation is critical for proper chromosome segregation.

Introduction

Post-translational modifications of histones, e.g., phosphorylation, methylation, ubiquitination, and acetylation, are critically involved in a number of cellular processes that range from regulating gene expression to repair of DNA damage [1–3]. Lysine acetylation of histones is controlled by histone acetyl transferases (HATs) and histone deacetylases (HDACs). In mammals, eighteen HDACs have been identified and grouped into four classes [4]. Class I enzymes HDAC1 and HDAC2 are highly homologous and ubiquitously expressed in different tissues [5]. HDAC1 and HDAC2 lack a DNA binding domain, as do all histone deacetylases, and execute their function by interacting with transcription factors as either homo- or heterodimers, or being part of multi-component repressor complexes [5].

Loss-of-function studies in mice have generated important insights regarding the function of HDAC1 and HDAC2 in regulating cell proliferation, apoptosis, and differentiation. One common theme of several tissue specific HDAC1/2 knockout studies is redundancy and compensation [6–9]. Nevertheless, results of other studies support the notion that HDAC1 and HDAC2 have distinct functions in some cells and tissues [10–13]. Taken together, these results indicate that the physiological functions of HDAC1 and HDAC2 are complicated and diversified in different tissues or cell types. Recently, we demonstrated compensatory functions of HDAC1 and HDAC2 during mouse oocyte development in which Hdac1−/− and Hdac2−/− were specifically deleted in oocytes [14]; deletion of both genes in oocytes results in infertility due to failure of follicle development beyond the secondary follicle stage with ensuing oocyte apoptosis attributed to hyperacetylation of TRP53.

We also noted in that study that deleting Hdac1−/− in oocytes has no effect on fertility, as also observed for mice harboring Hdac1−/−/Hdac2−/− or Hdac2−/− oocytes. In contrast, mice in which Hdac2−/− was only deleted in oocytes are sub-fertile despite increased amounts of HDAC1 protein whereas their Hdac1−/−/Hdac2−/− counterparts are infertile [14]. Taken together, these results suggest a more prominent role for HDAC2 than HDAC1 in oocyte development, whereas HDAC1 plays a more prominent role in preimplantation development [13]. The molecular basis for the sub-fertility of mice harboring Hdac2−/− oocytes or infertility of mice harboring Hdac1−/−/Hdac2−/−, however, was not examined.

We report here a characterization of the infertility and sub-fertility phenotypes observed in mice harboring Hdac1−/−/Hdac2−/− or Hdac2−/− oocytes, respectively. We find that full-grown or nearly full-grown oocytes can be obtained from mice containing Hdac2−/− or Hdac1−/−/Hdac2−/− oocytes, respectively, albeit highly reduced numbers of Hdac1−/−/Hdac2−/− oocytes are recovered. Oocytes derived from Hdac1−/−/Hdac2−/− mice are not capable of supporting development due to defects beyond failure to develop...
Oocyte development is becoming of increasing interest not only in the broad research community but also within the general public due, in part, to the ever increasing demand for and use of assisted reproductive technologies (ART) to treat human infertility, and because the oocyte-to-embryo transition encompasses a natural reprogramming of gene expression, a process central to forming iPS cells. Dramatic changes in chromatin structure and gene expression occur during oocyte development, but the role of such changes in generating oocytes that are capable of maturing, being fertilized, and giving rise to offspring is very poorly understood. Histone deacetylases (HDACs) are critically involved in modulating chromatin structure. Here, we describe the effect of specifically deleting the gene encoding Hdac2 in mouse oocytes and find the fertility of female mice harboring such oocytes is compromised. Although such mutant oocytes can grow they fail to mature properly to become an egg. The primary defect is that histone H4 acetylated on lysine 16 fails to become deacetylated as the oocyte matures to become an egg, with the consequence that the ability of chromosomes to interact with spindle microtubules is compromised, which in turn leads to improper chromosome segregation.

Demethylation but Not Apoptosis

Our previous study characterizing the phenotype of Hdac1<sup>−/−</sup>/Hdac2<sup>+/−</sup> oocytes was performed with 12-day-old mice [14]. Accordingly, we conducted studies regarding transcription and histone modifications using Hdac1<sup>−/−</sup>/Hdac2<sup>+/−</sup> oocytes collected on day-12 post-partum to make valid comparisons. Hdac2 transcripts were reduced by ∼95% in oocytes obtained from 12-day-old Hdac1<sup>−/−</sup>/Hdac2<sup>+/−</sup> mice, whereas Hdac1 mRNA level only decreased ∼15% (Figure 2A), which was reflected by a dramatic decrease in the nuclear staining of HDAC2 and only modest decrease (<15%) in HDAC1 nuclear staining (Figure 2B). The small decrease in Hdac1 mRNA likely reflects a compensatory increase in Hdac1 expression in face of loss of Hdac2 [14]. Similar to double mutant oocytes [14], there was an increase in acetylation of both histone H3 and H4 on specific lysine residues (Figure 2C and 2E).

Transcription was reduced by ∼20%, as assayed by EU incorporation, in Hdac1<sup>−/−</sup>/Hdac2<sup>+/−</sup> oocytes when compared to WT oocytes (Figure 2D and 2E), being intermediate when compared to Hdac1<sup>−/−</sup>/Hdac2<sup>−/−</sup> oocytes in which transcription is reduced by 40% [14]. Hdac2<sup>−/−</sup> oocytes did not exhibit any significant decrease in the extent of EU incorporation relative to WT (33±4 relative units vs 48±2 relative units, respectively; p=0.20. The data are expressed as mean ± SEM, and the number of oocytes examined was 16 and 24, respectively). We also observed a significant decrease in the intensity of H3K1me1&2 (∼20%) and H3K1me3 (∼40%) nuclear staining in Hdac1<sup>−/−</sup>/Hdac2<sup>+/−</sup> oocytes (Figure 2D and 2E); active promoters are marked in general by trimethylated H3K4 (H3K4me3), whereas dimethylated H3K4 (H3K4me2) is often found in the coding region [16,17]. A decrease in H3K1me1 of ∼20%, and a decrease in H3K4me2 and H3K1me3 of ∼35% and 60%, respectively were observed in Hdac1<sup>−/−</sup>/Hdac2<sup>−/−</sup> oocytes (Figure 3 and Fig. 6D in [14]).
using antibodies that specifically recognize CTD S2 phosphorylation, which is a marker for RNA polymerase II engaged in transcription [18], a significant reduction (~20%) was observed in CTD S2 phosphorylation in Hdac1\(^{-/-}\)/Hdac2\(^{-/-}\) growing oocytes (Figure 2D and 2E). This decrease was consistent with the 20% decrease in global transcription.

We previously observed a 40-fold increase in the amount of Kdm5b transcript in Hdac1\(^{-/-}\)/Hdac2\(^{-/-}\) oocytes and no change in Hdac1\(^{-/-}\) oocytes [14]; KDM5B is apparently the only histone lysine demethylase that can demethylate H3K4me3, H3K4me2 and H3K4me1 [19]. Kdm5b transcripts were also increased, as determined by qRT-PCR, by 18-fold in Hdac1\(^{-/-}\)/Hdac2\(^{-/-}\) oocytes and 3-fold in Hdac1\(^{-/-}\)/Hdac2\(^{-/-}\) oocytes. The extent of up-regulation of Kdm5b transcripts was related to the extent of loss of Hdac1 and Hdac2, with a corresponding decrease in histone H3K4me1-3 (Figure 3B). These results suggest that KDM5B plays a significant role in establishing the steady-state amount of methylated histone H3K4. In contrast to the dramatic increase in TRP53K379 acetylation that occurs in Hdac1\(^{-/-}\)/Hdac2\(^{-/-}\) oocytes [14] and results in increased TRP53 activity [20,21], TRP53 was not hyperacetylated in Hdac1\(^{-/-}\)/Hdac2\(^{-/-}\) oocytes (Figure 2D and 2E), consistent with apoptosis not being observed in oocytes from these mice. The absence of TRP53 acetylation suggests that only one allele of Hdac1 or Hdac2 is sufficient to prevent increased TRP53 activity in growing oocytes.

Depletion of Maternal HDAC2 Leads to Hyperacetylation of H4K16 and Defective Chromosome Condensation and Segregation during Oocyte Maturation

Although most Hdac1\(^{-/-}\)/Hdac2\(^{-/-}\) oocytes were arrested within secondary follicles (Figure 1D and 1E), a small number of nearly full-grown oocytes could be recovered from Hdac1\(^{-/-}\)/Hdac2\(^{-/-}\) oocytes (Figure 1B). A single mutant of Hdac1 or Hdac2 has no apparent effect on oocyte maturation and subsequent preimplantation development [14] and Figures S1 and S2). We next asked if depletion of HDAC2 combined with a reduction of HDAC1 has any effect on oocyte maturation by using Hdac1\(^{-/-}\)/Hdac2\(^{-/-}\) oocytes.

We first checked Hdac1 and Hdac2 expression in full-grown Hdac1\(^{-/-}\)/Hdac2\(^{-/-}\) oocytes. qRT-PCR revealed that Hdac2 mRNA was reduced by >98% in Hdac1\(^{-/-}\)/Hdac2\(^{-/-}\) full-grown oocytes (Figure 4A). Hdac1 mRNA levels, however, showed no difference between Hdac1\(^{-/-}\)/Hdac2\(^{-/-}\) and WT oocytes (Figure 4A), which suggests an ~2-fold compensatory increase in Hdac1 expression in Hdac1\(^{-/-}\)/Hdac2\(^{-/-}\) oocytes. Consistent with these results, the amount of HDAC1 protein exhibited a mild increase in Hdac1\(^{-/-}\)/Hdac2\(^{-/-}\) oocytes when compared to WT oocytes (Figure 4B), whereas there was a significant increase of both Hdac1 mRNA and HDAC1 protein in Hdac2\(^{-/-}\) oocytes (Figure 4A and 4B). Because the amount of HDAC1 present in Hdac1\(^{-/-}\)/Hdac2\(^{-/-}\) and Hdac2\(^{-/-}\) oocytes was similar to or more than that in WT oocytes, Hdac1\(^{-/-}\)/Hdac2\(^{-/-}\) and Hdac2\(^{-/-}\) oocytes provide a system to assess the role of maternal HDAC2 in oocyte maturation and subsequent fertilization and preimplantation development.

Genome-wide histone deacetylation mediated by HDACs at several lysine residues occurs during oocyte maturation in mouse [22,23,24] and pig [25]. The identity of the responsible HDACs, however, is poorly defined in mouse. Accordingly, we first assessed the effect of deleting maternal HDAC2 on histone acetylation during oocyte maturation. Remarkably, immunostaining revealed that although the amount of HDAC1 associated with chromosomes was unchanged in mutant oocytes, the acetylation state of...
histone H4K16 was affected, being increased 2.3-fold in Hdac2−/− and 4-fold in Hdac1−/−/Hdac2−/− MII eggs (Figure 4C and 4D); the maturation-associated deacetylation of histone H4K5, H4K8, H4K12, H3K4, H3K9, and H3K14 appeared to occur normally in these cells (Figure S3). It was unlikely that an increase in MYST1, an H4K16-specific histone acetyltransferase [26] and largely responsible for H4K16 acetylation [27], accounted for the observed increase in acetylated H4K16 following maturation because there was no obvious change in the amount of MYST1 protein in Hdac1−/−/Hdac2−/− or Hdac2−/− oocytes (Figure S4A). The amount of acetylated H4K16 was increased in growing Hdac1−/−/Hdac2−/− oocytes, suggesting nuclear HDAC2 can deacetylate H4K16 (Figure 2C and 2D). Nuclear HDAC2 in full-grown oocytes, however, appeared unable to deacetylate H4K16 because similar amounts of acetylated H4K16 were observed in full-grown WT and Hdac1−/−/Hdac2−/− oocytes (Figure S4B).

The increased acetylation of H4K16 following maturation of mutant oocytes was presumably due to loss of HDAC2 activity because Hdac2−/− oocytes injected with an Hdac2 cRNA, but not an Egfp cRNA, exhibited the maturation-associated decrease in acetylated H4K16 to a similar degree as observed in wild-type oocytes (Figure 5). Note that HDAC2 was expressed to similar levels in mutant oocytes when compared to wild-type oocytes.
minimizing the likelihood that deacetylation of H4K16 was due to off-targeting effects. In addition, chromosomes appeared less condensed in Hdac1−/−/Hdac2−/− and Hdac2−/− MII eggs; whereas only 2.4% of WT eggs (4 out of 163) had chromosomes that appeared not fully condensed, this incidence increased to 30.8% (28 out of 91) and 44.9% (40 out of 89) in Hdac2−/− and Hdac1−/−/Hdac2−/− MII eggs, respectively.

The effect of deleting Hdac1 and Hdac2 on the acetylation state of H4-K16 and chromosome condensation next led us to examine spindle formation and chromosome alignment in Hdac1−/−/Hdac2−/− and Hdac2−/− eggs following oocyte maturation. Analysis of meiotic spindle configurations in MII eggs revealed a representative images and only the nucleus is shown. The bar analyzed, and the experiment was conducted 3 times. Shown are

Figure 3. Loss of HDAC1/2 leads to H3K4me1-3 demethylation and up-regulation of KDM5B. (A) Immunocytochemical detection of H3K4me1, H3K4me2 and H3K4me3 in oocytes obtained from mice 12-days-of-age and lacking different combinations of Hdac1 and Hdac2. For each histone variant, at least 20 oocytes for each genotype were analyzed, and the experiment was conducted 3 times. Shown are representative images and only the nucleus is shown. The bar corresponds to 10 μm. (B) Quantification of the data shown in panel A and relative abundance of Kdm5b mRNA in different genotype oocytes obtained from mice 12-days-of-age. For immunofluorescence quantification, the staining intensity of H3K4me1-3 in the WT oocytes was set to 100. The relative abundance of Kdm5b transcript was assayed by qRT-PCR and expressed relative to WT Kdm5b mRNA level that was set as 1. UBF was used as internal control. All data are expressed as mean ± SEM. doi:10.1371/journal.pgen.1003377.g003

In somatic cells, histone hyperacetylation can interfere with kinetochore assembly [20] and in budding yeast, which have 125 bp “point” centromeres (which contrasts to 0.1–5 Mb bp “regional” centromeres in fission yeast and humans), hyperacetylation of H4K16 is critical to maintain kinetochore function [29]. Hyperacetylation of H4K16 in oocyte lacking HDAC2, therefore, could also compromise kinetochore function and lead to the increased incidence of misaligned chromosomes on the spindle. Such appears to be the case. Most spindle microtubules depolymerize at low temperature, except for kinetochore microtubules, which are preferentially stabilized [30], i.e., compromised kinetochore function leads to a decrease in the number of cold-stable microtubules. Finding that the incidence of kinetochores, as detected by CREST staining, not associated with cold-stable microtubules was significantly increased in mutant oocytes strongly implies that kinetochore function is compromised (Figure 8). This conclusion is further buttressed by observing less CENP-A staining at centromeres in mutant oocytes (Figure 9).

Hdac1−/−/Hdac2−/− Eggs Are Fertilized but Fail to Initiate DNA Replication

The incidence of aneuploidy in Hdac2−/− eggs could account for the observed sub-fertility in mutant female mice, but would not account for the infertility in Hdac1−/−/Hdac2−/− mice. Accordingly we ascertained whether Hdac1−/−/Hdac2−/− eggs could be fertilized, and if so assessed their ability to develop. Hdac1−/−/Hdac2−/− eggs were readily fertilized as evidenced by formation of a male and female pronucleus; only small fraction (~15%) of these eggs failed to be fertilized. When Hdac1−/−/Hdac2−/− female mice were mated to wild-type males, no blastocysts were recovered following development in vivo. Performing a similar mating in which 1-cell embryos were recovered and then permitted to develop in vitro demonstrated that most Hdac1−/−/Hdac2−/− embryos arrested at the 1-cell (53%) and 2-cell (~20%) stages, and a higher incidence of fragmentation was observed (Figure 10A).

The high incidence of failure of Hdac1−/−/Hdac2−/− 1-cell embryos to cleave to the 2-cell stage led us to examine whether failure to undergo DNA replication was the cause. Whereas BrdU was readily incorporated by zygotes obtained from WT and Hdac2−/− eggs, all zygotes obtained from Hdac1−/−/Hdac2−/− eggs failed to incorporate BrdU (Figure 10B). The 2-cell embryos observed following in vitro culture of Hdac1−/−/Hdac2−/− eggs, therefore, were likely the products of pseudo-cleavage. Last, BrdU incorporation by zygotes obtained from Hdac2−/− eggs suggests that the up-regulation of HDAC1 in Hdac2−/− oocytes compensates for loss of HDAC2.

Deleting both Hdac1 and Hdac2 in dividing cells results in a cell cycle block in G1 phase through induction of expression of the cyclin-dependent kinase inhibitors P21 and P57 [9]. Due to the cycle block in G1 phase through induction of expression of the cyclin-dependent kinase inhibitors P21 and P57 [9]. Due to the cycle block in G1 phase through induction of expression of the cyclin-dependent kinase inhibitors P21 and P57 [9]. Due to the cycle block in G1 phase through induction of expression of the cyclin-dependent kinase inhibitors P21 and P57 [9]. Due to the cycle block in G1 phase through induction of expression of the cyclin-dependent kinase inhibitors P21 and P57 [9]. Due to the cycle block in G1 phase through induction of expression of the cyclin-dependent kinase inhibitors P21 and P57 [9]. Due to the cycle block in G1 phase through induction of expression of the cyclin-dependent kinase inhibitors P21 and P57 [9]. Due to the cycle block in G1 phase through induction of expression of the cyclin-dependent kinase inhibitors P21 and P57 [9]. Due to the cycle block in G1 phase through induction of expression of the cyclin-dependent kinase inhibitors P21 and P57.
Immunoblot analysis of HDAC1 and HDAC2 expression in WT and mutant full-grown oocytes; total protein was extracted from fully-grown oocytes 6 times, and similar results were obtained in each case. ACTB was used as a loading control. (C) Immunocytochemical detection of HDAC1 and histone H4K16 acetylation following oocyte maturation. HDAC1 and histone H4K16 acetylation in WT, Hdac2−/− and Hdac1+/−/Hdac2−/− mice; at least 20 oocytes from each genotype were analyzed.

Figure 4. Depletion of maternal HDAC2 results in increased histone H4K16 acetylation following oocyte maturation. (A) Relative abundance of Hdac1 and Hdac2 transcripts in full-grown oocytes obtained from WT, Hdac2−/− and Hdac1+/−/Hdac2−/− mice. Data are expressed relative to that in WT oocytes. The experiment was performed four times and the data expressed as mean ± SEM. *, p<0.05; **, p<0.001. (B) Immunoblot analysis of HDAC1 and HDAC2 expression in WT and mutant full-grown oocytes; total protein was extracted from fully-grown oocytes (180 for HDAC1 and 80 for HDAC2) obtained from WT, Hdac2−/− and Hdac1+/−/Hdac2−/− mice for immunoblotting. The experiment was conducted 3 times, and similar results were obtained in each case. ACTB was used as a loading control. (C) Immunocytochemical detection of HDAC1 and histone H4K16 acetylation in WT, Hdac2−/− and Hdac1+/−/Hdac2−/− MII eggs; at least 20 oocytes from each genotype were analyzed, and the experiment was conducted 3 times. Shown are representative images and the DNA was stained with Sytox Green (green) or propidium iodide (Red). The bar corresponds to 10 μm. (D) Quantification of the data shown in panel C. Staining intensity of different proteins in WT eggs was set to 1 and the data are expressed as mean ± SEM. At least 20 eggs for each genotype and for each detected protein were analyzed; the experiment was conducted three times.* p<0.05; **, p<0.001.
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Discussion

We previously demonstrated that specifically deleting in oocytes both Hdac1 and Hdac2 genes led to developmental failure beyond the secondary follicle stage, the likely consequence of a 40% decrease in transcription, a massive perturbation in the transcriptome, and TRP53 hyperacetylation leading to apoptosis. The results reported here extend our understanding of HDAC1 and HDAC2 functions during oocyte development by identifying processes they control during oocyte maturation and following fertilization.

The ability of Hdac1−/−/Hdac2−/− or Hdac2−/− oocytes to reach the nearly full-grown or full-grown stage within preovulatory antral follicles is likely a consequence that they fail to undergo apoptosis because TRP53 is not hyperacetylated. Nevertheless, only a small fraction of Hdac1−/−/Hdac2−/− oocytes develop beyond the secondary follicle stage, whereas development beyond the secondary follicle stage is quite robust in Hdac2−/− oocytes. This difference may reflect that global transcription is reduced by 20% in Hdac1−/−/Hdac2−/− oocytes but unaffected in Hdac2−/− oocytes. The perturbation in transcription in Hdac1−/−/Hdac2−/− oocytes could impact the communication that exists between oocytes and the surrounding granulosa/cumulus cells and is essential for both oocyte growth and follicle cell proliferation [33]. Although not determined here, the transcriptome of Hdac1−/−/Hdac2−/− oocytes is likely dramatically perturbed as it is in double knock out oocytes [14]. That such an alteration could compromise follicle cell function, is supported by our finding that whereas ovarian weight increases ~2-fold following eCG priming of 21-day-old WT mice, ovaries from mutant mice fail to respond (data not shown). We also find that deleting Hdac2 results in an increased incidence of aneuploidy that is associated with hyperacetylation of H4K16 following oocyte maturation, a finding implicating HDAC2 in chromosome condensation and segregation. Last, depletion of HDAC2 combined with reduction of HDAC1 in Hdac1−/−/Hdac2−/− oocytes results in a subpopulation that can mature to MII, but following egg activation, fail to replicate their DNA.

There is no apparent effect on fertility of Hdac1−/−/Hdac2−/+ mice [14], whereas Hdac1−/−/Hdac2−/+ mice are infertile, their infertility attributed to a combination of defects in oocyte development, maturation, and embryo development. These results provide further support that HDAC2 plays a more prominent role during oocyte development, whereas HDAC1, which is zygotically expressed, is the major HDAC regulating preimplantation development [13]. HDAC1 function during oocyte maturation and following fertilization remains less defined. The amount of HDAC1 protein in Hdac1−/− full-grown oocytes is ~55% that of WT oocytes (Figure S1B), confounding analysis of HDAC1 function because such mice are fully fertile [14]. The highest levels of Hdac1 expression are in the primordial and primary...
follicle stages [14,34], which may explain why the 2b-Cre mediated strategy did not effectively deplete HDAC1; the 2b promoter becomes active during the primordial to primary follicle transition [35].

Oocyte growth is accompanied by a transition from the so-called non-surrounded nucleus (NSN) configuration in which condensed chromatin does not surround the nucleolus to surrounded nucleus (SN) configuration that is characterized by highly condensed chromatin around the nucleolus [36,37]. During this transition, which is uncoupled for the onset of transcriptional quiescence [22,38,39], there is an increase in both histone acetylation and methylation [40]. These increases that occur during oocyte growth are not affected in Hdac2 -/- oocytes (Figure S7A, S7B), i.e., full-grown mutant SN oocytes display the increase whereas mutant NSN oocytes do not.

Hdac1 +/- /Hdac2 -/- oocytes that develop beyond the secondary follicle stage and are capable of undergoing meiotic maturation display two phenotypes. One phenotype is a failure to condense fully chromosomes (see below for further discussion of HDAC2 in chromosome dynamics). For those oocytes that mature to MII, the phenotype is a failure to undergo DNA replication following insemination. Whether the failure to replicate DNA is directly linked to altered histone acetylation or a consequence of perturbed transcription during oocyte development/acetylation of non-histone proteins is unknown. In general, however, there is a positive relationship between histone acetylation and DNA replication ([41] and references therein), possibly a consequence of increased chromatin accessibility that facilitates pre-replicative complex assembly. Last, the observed early developmental arrest is similar to several maternal-effect mutants (e.g., Non2, Stella, Znr1, Hsf1, Mll2, and Mater) [38,39,42-45], which arrest primarily at the 1-2-cell stage. No DNA replication defects, however, were reported for these mutants, suggesting a different mechanism for developmental arrest.

Mouse oocyte maturation is accompanied by a global decrease in acetylated H3 and H4 histones [22-24], but the responsible HDACs have yet to be identified. Histone H3 and H4 deacetylation are not observed during M-phase for preimplantation embryos, except for deacetylation of H4K5 [23]. A similar situation is observed in NIH 3T3 cells, i.e., only H4K5 is deacetylated during M phase [23]. HDACs are clearly active in full-grown GV-intact oocytes because treatment with TSA, an HDAC inhibitor, results in increased histone acetylation [22]. Likewise, histone hyperacetylation is observed in growing oocytes in which Hdac1 and Hdac2 have been specifically deleted [14]. The amount of acetylated histone in GV-intact oocytes, therefore, represents a steady-state level reflecting HDAC and HAT activities. HDAC activity presumably outstrips HAT activity following maturation, thus accounting for the global decrease in histone acetylation. For example, treating MII eggs with TSA does not lead to an increase in histone acetylation, suggesting that HATs are inactive (or cannot access their histone substrates). In contrast, HDACs remain functional (or have access to their histone substrates) because oocytes matured in the presence of TSA fail to exhibit histone deacetylation but do so shortly following transfer to TSA-free medium [23].

HDAC2 is clearly implicated in the maturation-associated deacetylation of H4K16 because an increase in H4K16 is observed in Hdac2 -/- /Hdac2 -/- and Hdac2 -/- /Hdac2 -/- MII eggs, but not in Hdac1 -/- /Hdac2 -/- oocytes (Figure S7C). The apparent greater increase in acetylated H4K16 in Hdac1 -/- /Hdac2 -/- /Hdac2 -/- MII eggs when compared to Hdac2 -/- /Hdac2 -/- MII eggs (Figure 4C) also suggests a role for HDAC1 in controlling the acetylation state of H4K16, albeit less than that of HDAC2. It should be noted that HDAC2 is not associated with chromosomes in MII eggs [13,14] and the amount of chromosome-associated HDAC1 is similar in Hdac1 -/- /Hdac2 -/- and Hdac2 -/- /Hdac2 -/- MII eggs (Figure 4C). Because the total amount of HDAC1 is greater in Hdac2 -/- oocytes than in Hdac1 -/- /Hdac2 -/- oocytes, HDAC1 not associated with chromosomes may be responsible for the decreased amount of acetylated H4K16 in Hdac2 -/- eggs when compared to Hdac1 -/- /Hdac2 -/- eggs.

To date, only class III HDACs (sirtuins) have been shown to deacetylate specifically histone H4K16 [46]. Taken together, our results suggest that HDAC2 is the HDAC largely responsible for the maturation-associated deacetylation of H4K16, i.e., Class I HDACs are involved. This conclusion contrasts with the conclusion drawn from a study using porcine oocytes that Class I HDACs are not involved and was based on the inability of...
valproic acid, an inhibitor of Class I HDACs, to inhibit the maturation-associated decrease in histone acetylation [47]. That study, however, only assayed for the acetylation state of histone H4K8 and H3K14, and not H4K16, which could reconcile the different conclusions. Last, other acetylated lysine residues in H3 and H4 are deacetylated during maturation in both Hdac1<sup>+/−</sup> /Hdac2<sup>−/−</sup> or Hdac2<sup>−/−</sup> oocytes. The identity of the responsible HDACs remains to be determined.

A striking finding reported here is the increase in H4K16 acetylation in virtually all oocytes lacking HDAC2 following oocyte maturation and the associated failure of chromosomes to condense fully and align on the metaphase plate properly in a subpopulation; these failures presumably underlie the observed increased incidence in aneuploidy. That not all mutant oocytes show abnormal chromosome condensation and chromosome alignment despite an increase in H4K16 acetylation in all mutant oocytes is consistent with the finding that when global histone hyperacetylation is induced by treatment with TSA, a substantial fraction [but not all] of the maturing oocytes become euploid (~40%) [48]. Thus, even when the maturation-associated deacetylation of histones is inhibited and histone acetylation is increased, oocytes/eggs apparently have robust mechanisms to ensure proper chromosome segregation. This ability is consistent with the centrality of oocytes/eggs in reproduction, i.e., robust mechanisms to segregate properly chromosomes are an outcome of strong selective pressures to maintain reproductive fitness. The lower incidence of aneuploidy exhibited in eggs lacking HDAC2, when compared to TSA-treated oocytes, is consistent with mutant oocytes undergoing normal histone deacetylation, except for H4K16.

Acetylation of H4K16 inhibits formation of the higher order 30 nm chromatin structure, and loss of H4K16 shows defects equivalent to the loss of the H4 tails [49]. The increased acetylation of H4K16 in oocytes lacking HDAC2 is likely contributes to the failure of chromosomes to condense fully during oocyte maturation, despite deacetylation of other acetylated lysines. In addition, the increased incidence in mutant oocytes of kinetochores not able to interact with microtubules to form cold-stable microtubules is a functional assay confirming that kinetochore function is indeed compromised. The basis for compromised kinetochore function may reside in histone hyperacetylation interfering with proper kinetochore assembly [50] that leads to the observed decrease in CENP-A staining in mutant oocytes.

Although an age-dependent loss of cohesion can account for the majority of aneuploidies associated with increased maternal age [51], compromised histone deacetylation during maturation may be another source. For example, following maturation of mouse oocytes obtained from old mice, normal deacetylation of H4K8 and H4K12 do not occur, whereas H4K16 and H3K14 are properly deacetylated [48]. Likewise, less deacetylation of histone H4K12 occurs following maturation of oocytes obtained from older women when compared to younger women, and moreover, residual acetylation is correlated with misaligned chromosomes [52]. Thus, compromised histone deacetylation following oocyte maturation in general may result in an increased incidence of aneuploidy by leading to misaligned chromosomes on the meiotic spindle.

In summary, the results reported here provide further evidence for a critical role of HDAC2 in oocyte development and provide explanations for the infertility observed in Hdac1<sup>+/−</sup> /Hdac2<sup>−/−</sup> and sub-fertility in Hdac2<sup>−/−</sup> female mice. In particular, the results suggest that HDAC2 is largely responsible for deacetylation of H4K16 that occurs during oocyte maturation and that such deacetylation is critical for proper chromosome segregation.

Materials and Methods

Mouse Mutants and Collection of 1-Cell Embryos

Details for generating mutant mouse lines, histological analysis of ovaries, oocytes and embryos collection, RNA extraction and real time RT-PCR, immunostaining and immunoblot analysis are described in [14]. Hdac1 or Hdac2 mutants, in which the gene has only been deleted in oocytes, are referred to as Hdac1<sup>−/−</sup> or Hdac2<sup>−/−</sup>, respectively, Hdac1-Hdac2 mutants (double mutant) are referred to as Hdac1<sup>−/−</sup> /Hdac2<sup>−/−</sup>. Hdac1 heterozygotes-Hdac2 null oocytes are referred to as Hdac1<sup>+/−</sup> /Hdac2<sup>−/−</sup> and Hdac1 null-Hdac2 heterozygote oocytes are referred to as Hdac1<sup>−/−</sup> /Hdac2<sup>−/+</sup>. To obtain 1-cell embryos for BrdU incorporation assays, Hdac1<sup>−/−</sup> /
Figure 7. Expression of HDAC2 in Hdac2<sup></sup>-/- oocytes restores normal chromosome condensation and spindle formation in MII eggs.

(A) Spindle morphology and chromosome alignment in MII eggs. Mutant oocytes were injected with a cRNA (0.4 µg/µl) encoding either Egfp (Hdac2<sup></sup>-/-C) or Hdac2 (Hdac2<sup></sup>-/-OE) and incubated 24 h in CZB containing milrinone to prevent maturation; controls were wild-type oocytes injected with Egfp cRNA (WT-C). The oocytes were allowed to mature following transfer to milrinone-free medium overnight. MII eggs were fixed and stained with β-tubulin antibody (red); DNA was counterstained with Sytox green (green). Shown are representative images. The bar corresponds to 10 µm. (B) Frequency of abnormal spindles and abnormal chromosome condensation in WT-C, Hdac2<sup></sup>-/-C and Hdac2<sup></sup>-/-OE MII eggs. 104 WT-C eggs, 104 Hdac2<sup></sup>-/-C eggs and 76 Hdac2<sup></sup>-/-OE eggs were analyzed respectively. *, p<0.05, χ².

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Table 1. Incidence of abnormal spindles in Hdac mutant MII eggs.

| Genotype     | # MII with abnormal spindle | Total MII | % Abnormal MII |
|--------------|----------------------------|-----------|---------------|
| WT           | 1                          | 125       | 0.8           |
| Hdac<sup></sup>-/- | 12                         | 148       | 8.8*          |
| Hdac<sup></sup>-/-/Hdac1<sup></sup>-/+ | 21                         | 135       | 15.6**         |

Difference between WT and mutant is significant, *P<0.05, **P<0.01, χ². The difference between Hdac<sup></sup>-/- and Hdac<sup></sup>-/-/Hdac1<sup></sup>-/+ MII eggs is also significant, P<0.05.

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Table 2. Incidence of aneuploidy in Hdac1<sup></sup>-/+/Hdac2<sup></sup>-/- and Hdac2<sup></sup>-/- eggs.

| Genotype     | # Aneuploid | Total # | % Aneuploid |
|--------------|-------------|---------|-------------|
| WT           | 1           | 77      | 1.3         |
| Hdac2<sup></sup>-/- | 11           | 90      | 12.2*       |
| Hdac1<sup></sup>-/+/Hdac2<sup></sup>-/- | 11           | 60      | 18*         |

*p<0.01. The difference between Hdac1<sup></sup>-/+/Hdac2<sup></sup>-/- and Hdac2<sup></sup>-/- eggs is not significant.

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Hdac2−/− female mice were superovulated with the injection of 5 IU of PMSG, followed 48 hours later by 5 IU of human chorionic gonadotropin (hCG). The mice were then mated with B6D2F1/J males (Jackson Laboratory, Bar Harbor, ME) and 1-cell embryos collected 24 h post hCG administration. Cumulus cells were removed by a brief hyaluronidase treatment (3 mg/ml). All animal experiments were approved by the institutional animal use and care committee and were consistent with the National Institutes of Health (NIH) guidelines.

**cRNA Preparation and Microinjection**

Mouse Hdac2 cDNA was cloned in a PIVT plasmid using standard recombinant DNA techniques. To prepare cRNAs, plasmids were linearized, and capped mRNAs were generated by in vitro transcription using T7 mMESSAGE mMachine (Ambion) according to the manufacturer’s instructions. Following in vitro transcription, cRNA was polyadenylated using the PolyA Tailing Kit (Ambion). Synthesized cRNA was then purified using an MEGAclean Kit (Ambion), redissolved in RNase-free water, and stored at −80°C.

Full-grown oocytes were collected from mice of different genotypes and cultured in CZB medium [53] containing 0.2 mM IBMX in an atmosphere of 5% CO2 in air at 37°C. Microinjection of oocytes was performed as previously described [54]. Injections were done in 10-μl drops of modified Whitten’s medium [55] containing 15 mM HEPES, pH 7.2, 7 mM Na2HCO3, 10 μg/ml gentamicin, and 0.01% PVA containing 2.5 μM milrinone. Approximately 10 pl of EGfp or Hdac2 cRNA was injected into the cytoplasm of GV oocytes using a PLI-100 Pico-Injector (Harvard Apparatus, Holliston, MA) on the stage of a Nikon TE2000 microscope equipped with Hoffman optics and Narishige micromanipulators. Following microinjection, oocytes were cultured in CZB plus IBMX for 24 h, and then the injected oocytes were matured by washing and culturing them in IBMX-free CZB medium for 18 h.

**In Vitro Transcription Assay**

5-Ethynyl uridine (5EU) incorporation assays to detect transcription were performed as previously described [56]. Meiotically incompetent growing oocytes (or embryos) were cultured in the presence of 1 mM 5EU in CZB medium for oocytes or KSOM+AA for embryos [57] for 1 h and then fixed in 2% paraformaldehyde in PBS for 20 min at room temperature. 5EU incorporation into RNA was detected using Click-iT RNA Alexa Fluor 488 HCS Assay (Invitrogen) according to the manufacturer’s protocol. Fluorescence was detected on a Leica TCS SP laser-scanning confocal microscope. The intensity of fluorescence was quantified using ImageJ software (National institutes of Health) as previously described [58].
Hdac2 microscope at 0.4 m respectively. Images were collected with a spinning disk confocal anti-TUBB antibody to label kinetochores and microtubules, chemistry was performed with CREST autoimmune serum and before fixing in 2% paraformaldehyde for 20 min. Immunocyto- 

cellular DNA 1-cell embryos. (A) Embryos derived from Hdac1+/−/Hdac2−/− females crossed to WT males arrest early in development. Results are presented as % embryos (average) ± SEM from 3 independent experiments. Abbreviations: c, cell; F, fragmented. (B) Confocal images of WT, Hdac2−/−, and Hdac1+/−/Hdac2−/− 1-cell embryos in which the incorporation of BrdU was detected by immunocytochemistry. Shown are representative images and DNA was counterstained with Sytox green (green). The experiment was conducted twice and at least 12 embryos were analyzed for each group. Only one of the two pronuclei was in the focal plane for the Hdac1+/−/Hdac2−/− sample. The bar corresponds to 10 μm.

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Incorporation of bromodeoxyuridine (BrdU)

Assays were conducted as previously described [32] except the 1-cell embryos were cultured for 5–8 h rather than 30 min in KSOM medium supplemented with 1 μM BrdU (Sigma).

Monastrol Treatment, Kinetochore

Immunocytochemistry, and Chromosome Counting

Monastrol treatment, immunocytochemical detection of kinetochore and chromosome counting were performed as previously described [51]. Images were collected with a spinning disk confocal miroscope at 0.4 μm intervals to span the entire region of the spindle, using a 100×1.4 NA oil immersion objective as described before [51].

TUNEL Labeling Assay

TUNEL (TdT-mediated dUTP nick end labeling) assays were performed with an In Situ Cell Death Detection Kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer’s instructions.

Antibodies

The following antibodies were used for immunofluorescence and/or immunoblotting: anti-CDC6 rabbit polyclonal antibody (11640-IAP; Proteintech; IF, 1:200), anti-P21 mouse monoclonal antibody (556430, BD Pharmingen; IF, 1:100), anti-P57 rabbit monoclonal antibody (2372-1; Epitomics; IF, 1:200), anti-ORC6L rat monoclonal antibody (4737; Cell signaling; IF, 1:100), and anti-CENP-A rabbit monoclonal antibody (2048; Cell signaling; IF, 1:200). All the other antibodies used in this paper have been described previously [14].

Statistics

Experiments were performed at least three times and the values are presented as mean ± SEM. All proportional data were subjected to an arcsine transformation before statistical analysis. Statistics were calculated with Microsoft Excel software. A P-value of <0.05 was considered to be statistically significant.

Supporting Information

Figure S1 Depletion of HDAC1 in oocytes has little effect on oocyte maturation and preimplantation development. (A) Relative abundance of Hdac1 and Hdac2 transcripts in full-grown oocytes obtained from WT and Hdac1−/− mice. Data are expressed relative to that in WT oocytes. The experiment was performed four times and the data expressed as mean ± SEM. * p<0.05. (B) Total protein was extracted from 300 growing or full-grown oocytes obtained from WT, Hdac1−/−, and Hdac2−/− mice for immunoblotting. The experiment was performed two times and similar results were obtained for each experiment. Note that the amount of HDAC1 protein displays a decrease during oocyte growth. Thus, the decrease in the relative amount of HDAC1 in incompetent oocytes appears greater than in full-grown oocytes. (C) Similar numbers of ovulated eggs are obtained from WT and Hdac1−/− mice after hormonal stimulation. At least 6 mice of each genotype were used, and the average number of ovulated oocytes per female is indicated. (D) Tubulin immunofluorescence staining of WT and Hdac1−/− MII eggs exhibiting normal spindles in both oocytes (upper panel). DNA was counterstained with Sytox green. The bar corresponds to 10 μm. One-cell embryos were collected from WT and Hdac1−/− mice and cultured 96 h in KSOM; representative bright-field photographs were showed in lower panel. The experiment was conducted 3 times. At least 100 embryos from each genotype were analyzed. The bar corresponds to 80 μm.

(TIF)

Figure S2 Effects of HDAC2 depletion in oocytes on HDAC1 and HDAC2 expression and preimplantation development. (A) Representative bright-field photographs from an embryo culture experiment. Maternally depleted HDAC2 and WT 1-cell embryos were collected 20 h after HCG injection then cultured for 24 h, 48 h, 72 h and 96 h. The bar corresponds to 80 μm. (B) Different stages of oocytes and embryos were collected from HDAC2 depleted and WT mice and were processed for immunocyto-
chemical staining of HDAC1 and HDAC2. Strong nuclear staining of HDAC1 was found in HDAC2 depleted GV oocytes, 1-cell and 2-cell embryos. At least 20 oocytes/embryos from each genotype at the indicated times were analyzed, and the experiment was conducted 3 times. Shown are representative images. The bar corresponds to 40 μm. (TIF)

Figure S3 Deletion of maternal HDAC2 has little effect on other histone lysine acetylation following oocyte maturation. Immunocytochemical detection of histone H3K4, H3K9 and H3K14 acetylation (A) or histone H4K3, H4K8 and H4K12 acetylation (B) in WT, Hdac2−/− and Hdac1−/−/Hdac2−/− MII eggs; at least 20 oocytes from each genotype were analyzed, and the experiment was conducted 2 times. Shown are representative images and the DNA was stained with Sytox Green. The bar corresponds to 10 μm. (TIF)

Figure S4 Deletion of HDAC2 has no effect on the expression of MYST1 and the acetylation state of H4K16 in WT, Hdac2−/− and Hdac1−/−/Hdac2−/− full-grown oocytes; at least 20 oocytes from each genotype were analyzed, and the experiment was conducted 3 times. Shown are representative images and the DNA was stained with Sytox Green. The bar corresponds to 10 μm. (B) Immunocytochemical detection of MYST1 in WT, Hdac2−/− and Hdac1−/−/Hdac2−/− full-grown oocytes; at least 20 oocytes from each genotype were analyzed, and the experiment was conducted 2 times. Shown are representative images and the DNA was stained with Sytox Green (green). The bar corresponds to 10 μm. (C) Immunocytochemical detection of ORC6L and CDC6 in WT and Hdac1−/−/Hdac2−/− 1-cell embryos. At least 20 embryos from each genotype were analyzed, and the experiment was conducted 2 times. Shown are representative images. The bar corresponds to 35 μm. (TIF)

Figure S5 Deletion of maternal HDAC2 causes aneuploidy in mouse eggs. MII eggs from WT, Hdac2−/− and Hdac1−/−/Hdac2−/− hormone-primed mice were treated with monastral to disperse the chromosomes, and then fixed and stained for DNA (Sytox, green) and kinetochores (CREST, red). Shown are representative images. The bar corresponds to 5 μm. WT egg is euploid with 20 paired sister kinetochores (numbered 1–40), whereas, the final kinetochore count in Hdac2−/− and Hdac1−/−/Hdac2−/− eggs is 38 and 37 respectively. (TIF)

Figure S6 Deletion of maternal HDAC2 results in 1-cell embryos block in G1 phase independent of P57 and P21. (A) Up-regulation of CDK inhibitors p21WAF1/CIP1 and p57Kip2 in Hdac1−/−/Hdac2−/− 1-cell embryos. The relative abundance of G1 phase specific CDK inhibitors transcripts was assayed by qRT-PCR and expressed relative to their WT mRNA levels. UBF was used as internal control. The experiment was performed 3 times and the data expressed as mean ± SEM. * p<0.05. (B) Immunocytochemical detection of P57 and P21 in WT and Hdac1−/−/Hdac2−/− 1-cell embryos. At least 20 embryos from each genotype were analyzed, and the experiment was conducted 2 times. Shown are representative images. The bar corresponds to 35 μm. (TIF)

Figure S7 Deletion of HDAC2 has little effect on histone modifications in NSN or SN full-grown oocytes. Immunocytochemical detection of histone acetylated H4K5, H4K8, H4K12 and H4K16 (A) or histone H3K4me1, H3K4me2 and H3K4me3 (B) in NSN or SN WT or Hdac2−/− oocytes. At least 20 oocytes from each genotype were analyzed, and the experiment was conducted 2 times. Shown are representative images and the DNA was stained with Sytox Green (green). The bar corresponds to 10 μm. (C) Immunocytochemical detection of Hdac1−/−/Hdac2−/− acetylation in WT, Hdac2−/− and Hdac1−/−/Hdac2−/− MII eggs; at least 20 oocytes from each genotype were analyzed, and the experiment was conducted 2 times. Shown are representative images and the DNA was stained with Sytox Green (green). The bar corresponds to 10 μm. Because acetylated histone H4K16 is extensively deacetylated during maturation, the laser power was increased so that the signal could readily be detected. (TIF)

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Author Contributions

Conceived and designed the experiments: PM RMS. Performed the experiments: PM. Analyzed the data: PM RMS. Wrote the paper: PM RMS.

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