Centromere-specifying nucleosomes persist in aging mouse oocytes in the absence of nascent assembly

Highlights

- Genetic removal of Mis18α tests current models for centromere inheritance in oocytes
- Mis18α removal has no impact on CENP-A nucleosome levels in aging oocytes
- Mis18α removal disrupts CENP-A nucleosome assembly in the early embryo
- CENP-A assembled prior to birth is sufficient for fertility in aging mice

Authors

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In brief

To test whether centromeres are maintained by new assembly in aging mouse oocytes, Das et al. conditionally delete Mis18α, a centromere assembly component. They find that centromere-specifying nucleosomes containing the histone H3 variant, CENP-A, are stably maintained even after 6–8 months of aging, with no detriment to fertility.
Centromere-specifying nucleosomes persist in aging mouse oocytes in the absence of nascent assembly

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SUMMARY

Centromeres direct genetic inheritance but are not themselves genetically encoded. Instead, centromeres are defined epigenetically by the presence of a histone H3 variant, CENP-A.1 In cultured somatic cells, an established paradigm of cell-cycle-coupled propagation maintains centromere identity: CENP-A is partitioned between sisters during replication and replenished by new assembly, which is restricted to G1. The mammalian female germ line challenges this model because of the cell-cycle arrest between pre-meiotic S phase and the subsequent G1, which can last for the entire reproductive lifespan (months to decades). New CENP-A chromatin assembly maintains centromeres during prophase I in worm and starfish oocytes,2,3 suggesting that a similar process may be required for centromere inheritance in mammals. To test this hypothesis, we developed an oocyte-specific conditional knockout (cKO) mouse for Mis18α, an essential component of the assembly machinery. We find that embryos derived from Mis18α knockout oocytes fail to assemble CENP-A nucleosomes prior to zygotic genome activation (ZGA), validating the knockout model. We show that deletion of Mis18α in the female germ line at the time of birth has no impact on centromeric CENP-A nucleosome abundance, even after 6–8 months of aging. In addition, there is no detectable detriment to fertility. Thus, centromere chromatin is maintained long-term, independent of new assembly during the extended prophase I arrest in mouse oocytes.

RESULTS AND DISCUSSION

In contrast to genetic information encoded in our genome, minimal epigenetic information is inherited because most parental epigenetic marks are removed and reprogrammed in germ cells and the early embryo.5,6 A key exception is the centromeric histone H3 variant, CENP-A.5,6 Although centromeres direct the process of genetic inheritance by connecting chromosomes to spindle microtubules, they are not encoded in DNA but rather epigenetically specified by nucleosomes containing CENP-A.1,9–11 Thus, CENP-A nucleosomes are inherited to preserve centromere identity.

Studies in cycling somatic cells have established a general paradigm for propagation of epigenetic information between cell cycles. DNA or histone modifications are partitioned between sister chromatids during DNA replication and then replenished by “reader” proteins that recognize the modification and “writers” that extend it to adjacent nucleosomes.12 CENP-A follows this paradigm, with new assembly restricted to G1 by CDK1/2 activity.13–15 CENP-A and its bound partner histone H4 molecules are remarkably stable in tissue culture cells,16,17 with measured CENP-A turnover rates explained entirely through the dilution when existing CENP-A is partitioned to replicated centromeric DNA during S phase in each cell cycle.16–20 This paradigm poses a challenge in the mammalian female germ line because of the extended prophase I cell-cycle arrest after replication but before an opportunity for new G1 assembly, which can last for the entire reproductive lifespan of the animal21 (Figure 1A). Centromeres are preserved throughout the arrest (>1 year in mouse) in the absence of new Cenpa transcription, as shown by conditional knockout (cKO) of the Cenpa gene.22 CENP-A nucleosomes are, therefore, either replenished by new assembly, in contrast to the somatic cell paradigm, drawing on a stable pool of CENP-A protein, or stable for the entire duration of the arrest (Figure 1B).

Studies in worm and starfish oocytes show nascent CENP-A chromatin assembly in prophase I (akin to a G2 biochemical cell-cycle state in a somatic cell),2,3 in mouse, continual deposition of nucleosomes containing another H3 variant, H3.3, during oocyte development is required for oocyte genome integrity and, ultimately, for fertility.24 These studies suggest that new assembly may maintain CENP-A chromatin through the prophase I arrest. To test this prediction, we created an oocyte-specific cKO of an essential component of the CENP-A deposition
machinery, Mis18α (Mis18a in mouse but referred to as Mis18α for simplicity), to prevent nascent CENP-A chromatin assembly. Mis18α is part of the Mis18 complex, which recruits the CENP-A chaperone, HJURP, bound to nascent CENP-A/histone H4 dimers, to centromeres. The Mis18 complex is required for both nascent CENP-A chromatin assembly in G1 and replication-coupled CENP-A chromatin assembly in S phase in cycling somatic cells. Specifically relevant to our test of ongoing chromatin assembly in the mouse oocyte, prophase I assembly in both worms and starfish oocytes requires the Mis18 complex. Thus, if CENP-A chromatin is replenished by new assembly, CENP-A nucleosome levels would decay in Mis18α KO oocytes.

For cKO, we used a floxed Mis18α allele in which the first two exons, encoding the YIPPEE domain necessary for CENP-A deposition, are flanked by loxP sites (floxed [fl]). The FRT site is a remnant from FLP-mediated excision of the neomycin cassette in the original construct used to generate the KO animals (see Figure S1 for genotyping).
prophase I (Figure S2). The early Cre driver (Gdf9-Cre) deletes Mis18α in arrested oocytes 2 days post birth, preventing assembly of new CENP-A nucleosomes for nearly the entire lifespan of the animal.37 The late Cre driver (Zp3-Cre) deletes the gene during oocyte growth, 2–3 weeks prior to ovulation37 (Figure 2A).

As a functional assay for Mis18α deletion, we tested for CENP-A chromatin assembly in embryos from late KO mothers (driven by Zp3-Cre) and a wild-type (WT) father (Figure 2B). Prior to zygotic genome activation (ZGA) at the two-cell stage of embryos, CENP-A deposition during the early embryonic mitotic cycles depends solely on maternal Mis18α protein. To assess new chromatin assembly, we injected mRNAs encoding CENP-A-EGFP (enhanced green fluorescent protein) and H2B-mCherry into one-cell embryos derived from either KO mothers or WT mothers as controls (Figure 2B). H2B-mCherry serves as a positive control for chromatin assembly because it utilizes a deposition pathway distinct from that of CENP-A and, therefore, does not require the Mis18 complex. Based on the paradigm established in cycling somatic cells, we expected new CENP-A chromatin assembly in G1 after the first embryonic mitosis. Any Mis18α protein present in the embryo prior to ZGA would be solely contributed maternally from the oocyte. We expect that embryos derived from control oocytes would be assembly proficient, whereas embryos from KO oocytes lacking Mis18α A

Table shows frequencies of detectable assembly for CENP-A-EGFP and H2B-mCherry, obtained from 2 independent matings. Scale bars, 5 μm. Also see Figures S2 and S3.

Figure 2. Maternally deposited Mis18α protein is eliminated in late KO oocytes
(A) Schematic showing early and late Mis18α knockout, depending on the timing of Cre recombinase expression driven by either Gdf9 or Zp3 promoters (colored arrows).
(B) Experimental design to test for a stable pool of Mis18α protein in the maternal cytoplasm. CENP-A-EGFP and H2B-mCherry mRNA are injected into one-cell embryos, and CENP-A foci are assessed after the first embryonic mitosis when assembly is expected to occur but before zygotic genome activation.
(C) Images show H2B-mCherry, CENP-A-EGFP, and DNA (4',6-diamidino-2-phenylindole [DAPI]) in interphase two-cell embryos from control or cKO mothers.

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would fail to assemble. Indeed, CENP-A-EGFP localized to centromeres in all control embryos (100%, n = 19) from WT mothers. In contrast, none of the embryos from late Mis18α KO mothers (0%, n = 20) contained detectable centromeric CENP-A-EGFP (Figure 2C). H2B-mCherry was present in chromatin in 100% of both control and KO-injected embryos, as expected. The all-or-none effect of Mis18α KO is due to the high efficiency of Cre excision and the relatively fast turnover of Mis18α mRNA in oocytes following excision (Figure S3). This finding establishes that even after a relatively short-duration KO, Mis18α deletion in oocytes abrogates nascent CENP-A chromatin assembly in early embryos.

Next, we measured fertility of Mis18α KO mothers as the ultimate test of centromere function because centromeres are required for the meiotic divisions and for maternal centromere inheritance, and even partial reduction of maternal CENP-A chromatin lowers fertility. All Mis18α early and late KO mothers were fertile when crossed to WT males (Figure 3A). We also confirmed that both Cre recombinases have a 100% deletion efficiency, as we did not recover a floxed allele of Mis18α inherited from a cKO mother (Figures 3B and 3C). Combined with our embryo experiments, this result confirms that every oocyte with a combination of floxed alleles and Cre lacks Mis18α protein and that fertility is not a consequence of inefficient Cre activity in some oocytes. Thus, centromere identity is maintained in aging oocytes without Mis18α protein or nascent CENP-A assembly.

Even though the cKO mice are fertile, there could still be a reduction in CENP-A nucleosomes over time. Therefore, we tested whether CENP-A levels decay in the late KO oocytes. We predicted that if CENP-A nucleosomes are continually replenished by new assembly from a stable pool of CENP-A protein, centromeric CENP-A levels would decline in the KO oocytes compared with control oocytes. We did not see any reduction in centromeric CENP-A levels in late KO oocytes compared with controls (Figures 4A and 4B). However, Mis18α is deleted for only 2–3 weeks before ovulation in the late KO, leaving open the possibility of a more dramatic reduction in CENP-A nucleosomes on longer timescales.

Thus, we leveraged the early KO (Gdf9-Cre) oocytes that delete Mis18α shortly after birth, to test whether centromeric

Figure 3. Mis18α knockout mothers can support fertility
(A) Litter sizes for control, early cKO, and late cKO mothers (age 2–4 months) crossed to wild-type (WT) males. See Figure S2 for generating cKO mothers. (B and C) Genotype frequencies of the progeny from a cross between a WT or Mis18αfl/fl father and an early or late cKO mother, respectively (N = 29 pups, 5 litters; N = 30 pups, 5 litters).
CENP-A levels decline after months without new assembly (Figures 4C and 4D). We aged control and early KO females for 6–8 months, representing most of the fertile lifespan, and found that CENP-A levels were not significantly reduced compared with the controls. Although not statistically significant, we measured a ∼3% decrease in CENP-A levels in the KO oocytes relative to the control (Figure 4D), which equates to loss at a rate of ∼0.02% per day over 180 days. Such loss in signal would be in the range of one nucleosome per month, assuming 200 CENP-A nucleosomes per centromere as estimated in human cells. In comparison, new assembly in starfish oocytes is estimated at a rate of 2% per day, based on centromere localization of GFP-tagged exogenous CENP-A in cells cultured in vitro for 10 days. Our result is consistent with our previous study, where we conditionally deleted the Cenpa gene using Gdf9-Cre and followed CENP-A nucleosome stability further and measured no substantial decrease.

Conclusions
In conclusion, these results provide clear evidence supporting long-term retention of CENP-A nucleosomes assembled prior to birth as the dominant pathway for maintaining centromere identity in mammalian oocytes. This is in stark contrast to H3.3, whose ongoing deposition into bulk chromatin during prophase I is essential for normal oocyte chromatin structure and fertility. In addition, our findings have implications for human female meiosis, which is inherently error prone and especially vulnerable to aging. With advancing maternal age at childbirth, mechanisms that preserve centromeres in aging oocytes gain increasing significance. Although maternal KO of Mis18α in mice preserves fertility due to early ZGA, maternal depletion of Mis18α is expected to have more severe consequences in human embryos, where activation occurs later. Our findings can now direct future research into the mechanisms that underlie CENP-A retention in mammalian oocytes. Previous studies of centromere chromatin suggest multiple potential molecular mechanisms that could contribute to its stability: the relatively low level of transcription of centromeric DNA relative to genic regions harboring histone H3.3 nucleosomes, structural features that differ from canonical nucleosomes—including internal structural rigidity at the CENP-A/histone H4 interface—and non-histone constitutive centromere associated network (CCAN) components that bind and stabilize CENP-A nucleosomes in tissue culture cells. In sum, it remains to be seen whether the mechanisms that function to retain CENP-A chromatin over short periods of time in cycling cells also contribute to extreme stability during oogenesis.

STAR METHODS
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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2023.07.032.

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AUTHOR CONTRIBUTIONS

A.D., M.A.L., and B.E.B. designed the project and wrote this manuscript, with input from co-authors. M.A.L. and B.E.B. provided supervision and sourced funding. M.A.L., B.E.B., and K.T. conceived the project. A.D. performed all the experiments and analyzed the data. A.D. and K.G.B. maintained the mouse input from co-authors. M.A.L. and B.E.B. provided supervision and sourced A.D., M.A.L., and B.E.B. designed the project and wrote this manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

REFERENCES


# KEY RESOURCES TABLE

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**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ben E. Black (blackbe@pennmedicine.upenn.edu).

**Materials availability**
Mouse lines generated in this study are available upon request.

**Data and code availability**
All imaging data reported in this paper will be shared by the lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS**

All animal experiments and protocols were approved by the Institutional Animal Use and Care Committee of the University of Pennsylvania and were consistent with National Institutes of Health guidelines (protocol #803994). Experimental animals were compared to age, gender and genetic background matched controls. The Mis18a<sup>fl/fl</sup> strain is a previously published and validated knockout strain.23 This strain is in a mixed genetic background of C57BL6/J/129Sv/CBA/J (generated by Sung Hee Baek, Seoul National University and obtained from Kikue Tachibana, Max Planck Institute). Oocyte specific conditional knockout strains Mis18a<sup>fl/fl;Zp3-Cre</sup> (Mis18<sup>late KO</sup>) and Mis18a<sup>fl/fl;Gdf9-iCre</sup> (Mis18<sup>early KO</sup>) were generated by crossing the original Mis18a<sup>fl/fl</sup> strain to either C57BL/6-Tg(Zp3-cre)93Knw/J (RRID:IMSR_JAX:003651, Jackson Laboratory) or Tg(Gdf9-icre)5092Coo/J (RRID:IMSR_JAX:011062, Jackson Laboratory). The following primers were used to genotype the animals: 1) Mis18a<sup>fl/fl</sup>: SDL-Forward: TGC CTA TTG GTG TAC CTT CCA GTG, LOXP-Reverse: CCT AAG TCG TTG ACC TAG ACT TTC AAC combined with SDL-Forward (Figure S1). For testing fertility, age matched single conditional knockout (Mis18<sup>late or early KO</sup>) or control mothers (Mis18<sup>fl/fl</sup>) were mated in cages to single males (either Mis18a<sup>fl/fl</sup> or Mis18a<sup>fl/fl</sup>). Litter sizes were determined for multiple mating pairs per cross. Oocytes and embryos were collected from multiple mothers for all experiments.

**METHOD DETAILS**

**Oocyte collection, meiotic maturation, and culture**
Female mice were hormonally primed with 5 U of pregnant mare serum gonadotropin (PMSG, Peptides International) 44-48 h before oocyte collection. Germinal vesicle intact oocytes were collected in bicarbonate-free minimal essential medium (M2, Sigma),
denuded from cumulus cells, and cultured in Chatot–Ziomek–Bavister\textsuperscript{18} (CZB, Fisher Scientific) medium covered with mineral oil (Sigma, BioXTRA) in a humidified atmosphere of 5% CO\textsubscript{2} in air at 37 °C. During collection, meiotic resumption was inhibited by addition of 2.5 mM milrinone (Sigma). Milrinone was subsequently washed out to allow meiotic resumption and oocytes were fixed 6–7 h later at metaphase I.

**Oocyte immunocytochemistry**
Oocytes were fixed in 2% paraformaldehyde (Sigma) in phosphate buffered saline (PBS) with 0.1% Triton X-100 (Sigma), pH 7.4, for 20 min at room temperature (r.t.), permeabilized in PBS with 0.2% Triton X-100 for 15 min at r.t., placed in blocking solution (PBS containing 0.3% bovine serum albumin (BSA) and 0.01% Tween-20) for 20 minutes at r.t., treated with λ-phosphatase (1,600 U, NEB) for 1 h at 30 °C for CENP-A staining, incubated for 1 h with primary antibody in blocking solution, washed three times for 10 min each, incubated for 1 h with secondary antibody, washed three times for 10 min each, and mounted in Vectashield with 4',6-diamino-2-phenylindole (DAPI; Vector) to visualize the chromosomes. The primary antibody was rabbit anti-mouse CENP-A (1:200, Cell Signaling, C51A7). The secondary antibody was donkey anti-rabbit Alexa Fluor 488 (1:500, Invitrogen).

**Microscopy**
Confocal images were collected as z-stacks with 0.5-μm intervals, using a microscope (DMI4000 B; Leica) equipped with a 63x 1.3-NA glycerol-immersion objective lens, an x-y piezo Z stage (Applied Scientific Instrumentation), a spinning disk confocal scanner (Yokogawa Corporation of America), an electron-multiplying charge-coupled device camera (ImageEM C9100-13; Hamamatsu Photonics) and either an LMM5 (Spectral Applied Research) or Versalase (Vortran Laser Technology) laser merge module, controlled by MetaMorph software (Molecular Devices, v7.10.3.294). Images were acquired using the same laser settings and all images in a panel were scaled the same. Single channels are shown wherever quantifications were performed.

**Embryo collection, microinjection and culture**
*Mis18a\textsuperscript{fl/fl} or Mis18a\textsuperscript{fl/+},Zp3-Cre (Mis18α late KO) females* were hormonally primed with 5 U of PMSG (Peptides International) and the oocytes were matured in vivo with 5 U of human chorionic gonadotropin (hCG; Sigma) before mating with B6D2F1/J males (F1 hybrid of a cross between C57BL6/J and DBA2/J; RRID:IMSR_JAX:100006, Jackson Laboratory). Males were fed a special low soymeal diet (5LG4 irradiated diet, Labdiet) and housed singly. Embryos were collected 14–16 h post hCG in M2 containing hyaluronidase (Sigma, BioXTRA) in a humidified atmosphere of 5% CO\textsubscript{2} in air at 37 °C.

**Embryos**
Embryos were then subject to inter-cytoplasmic microinjection in M2 medium covered with mineral oil (Sigma, BioXTRA) at r.t. with a micromanipulator (Narishige) and a picoinjector (Medical Systems Corp). Each embryo was injected with 2 pl of cRNA, then cultured in EmbryoMax Advanced KSOM (AKSOM, Millipore Sigma) with humidified air and 5% CO\textsubscript{2} until 2 cell stage and fixed in 2% paraformaldehyde. The following cRNAs were used for microinjection: H2B-mCherry (human histone H2B with mCherry at the C-terminus) at 25 ng/ul, CENP-A-EGFP (mouse CENP-A with EGFP at the C-terminus) at 20 ng/ul. The cRNAs were synthesized using the T7 mScript Standard mRNA kit (Thermo Fisher Scientific) and purified by phenol-chloroform extraction.

**mRNA quantification in oocytes**
Total RNA was extracted from at least 20 full-grown oocytes from two females each for control and late conditional knockout (Zp3-Cre mediated) using Arcturus Picopure RNA isolation kit (Thermofisher Scientific), and cDNA was prepared by reverse transcription of total RNA with Superscript III First Strand Synthesis system (Thermofisher) using oligo dT primers. *Mis18α* was amplified for standard PCR using Kapa polymerase (Roche) from 1 μg of cDNA. Real time PCR was performed using *Mis18α* Taqman probes and H2A serving as the endogenous control. Each sample was run twice in triplicate. Quantification was performed using the comparative Ct method (Livak method) on an Applied Biosystems ViiA 7 machine.

**QUANTIFICATION AND STATISTICAL ANALYSIS**
To quantify centromere signal ratios, a sum intensity Z-projection was made using Fiji/ImageJ software. Circles of constant diameter were drawn around individual centromeres and the average intensity was calculated for each centromere after subtracting back­ground, obtained from nearby regions. Raw centromere intensities were obtained from several controlled independent experiments and multiple cells were analyzed from each animal. Normalization of centromere intensities was performed using age- and gender-matched controls for each independent experiment. Statistical tests (Mann-Whitney U test) were performed using the Graphpad Prism software. Details of the p-values and error bars are provided in figure legends.