

Long-Term Retention of CENP-A Nucleosomes in Mammalian Oocytes Underpins Transgenerational Inheritance of Centromere Identity

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SUMMARY

Centromeres control genetic inheritance by directing chromosome segregation but are not genetically encoded themselves. Rather, centromeres are defined by nucleosomes containing CENP-A, a histone H3 variant [1]. In cycling somatic cells, centromere identity is maintained by an established cell-cycle-coupled CENP-A chromatin assembly pathway, but how centromeres are inherited through the mammalian female germline is unclear because of the long (months to decades) prophase I arrest. Here we show that mouse oocytes retain the pool of CENP-A nucleosomes assembled before birth, and that this pool is sufficient for centromere function, fertility, and genome transmission to embryos. Indeed, oocytes lack any measurable CENP-A nucleosome assembly through the entire fertile lifespan of the female (>1 year). Thus, the remarkable stability of CENP-A nucleosomes confers transgenerational centromere identity in mammals.

RESULTS AND DISCUSSION

A pathway for centromere inheritance between somatic cell cycles is well established [1]. CENP-A nucleosomes redistribute equally between sister centromeres during DNA replication, and newly synthesized CENP-A is assembled at human centromeres exclusively in early G1 phase of the cell cycle [2, 3]. Therefore, centromere inheritance depends on retention of CENP-A nucleosomes from incorporation into chromatin in S phase until new loading in the subsequent G1 phase. This cell-cycle-coupled mechanism of centromere maintenance raises the question of how centromeres are inherited between generations in the mammalian female germline. Between pre-meiotic S phase in the oocyte and the subsequent G1 phase in the zygote, mammalian oocytes arrest in an extended prophase I that may last from a few months to decades depending on species [4]. Three attractive models could explain how centromere identity is maintained during this meiotic arrest: (1) CENP-A is not retained at centromeres during female meiosis, and centromere

identity in the germline is independent of CENP-A; (2) CENP-A is maintained through the action of a meiotic chromatin assembly pathway, which is distinct from the timing of the established cell-cycle-coupled pathway in cycling somatic cells; or (3) CENP-A nucleosomes assembled at oocyte centromeres are extremely stable and maintain centromere identity in the absence of any nascent CENP-A chromatin assembly during the prolonged arrest. The first two models have strong precedent in holocentric worms [5, 6] and flies [7], respectively. The third model is supported by the notable stability of CENP-A nucleosomes in chromatin: they do not measurably redistribute between centromeres during an entire cell cycle, and their turnover in rapidly dividing somatic mammalian cells is so slow that it can be explained by dilution through segregation on daughter strands of replicating centromere DNA [2, 3, 8]. We use mouse as a model system to distinguish between these models for centromere inheritance in the mammalian female germline.

We first tested for a meiotic CENP-A chromatin assembly pathway on a short timescale in full-grown germinal vesicle intact (GV) oocytes by injecting cRNAs encoding fluorescently tagged proteins: CENP-A; the histone H3 variant H3.3; or CENP-C, which binds CENP-A nucleosomes at centromeres [2, 9] (Figure 1A). We find that H3.3-mCherry assembles into chromatin, as previously shown in oocytes [10, 11], and GFP-CENP-C targets to centromeres, but no CENP-A-GFP is detected at centromeres either in prophase of meiosis I (GV) or after maturation to metaphase II (MII) (Figures 1B and 1C), though we do detect a measureable increase in GFP fluorescence in oocytes injected with CENP-A-GFP cRNA (Figure S1A). The CENP-A-GFP cRNA produced functional protein capable of assembly at centromeres, as shown by co-localization of CENP-A-GFP and mCherry-CENP-C in four-cell embryos (Figures 1D and 1E). We obtained similar results using CENP-A tagged with Flag and hemagglutinin (HA) epitopes instead of GFP (Figures S1B and S1C). These data show that a meiosis-specific loading pathway does not assemble measurable CENP-A in full-grown oocytes on a timescale of ~40 hr.

To test for CENP-A nucleosome assembly on timescales of many months during the prophase I arrest, we designed a genetic experiment in which we knocked out *Cenpa* in oocytes in resting primordial follicles (Figure 2A). Oocytes in these animals would rely on CENP-A nucleosomes that were assembled at centromeres prior to the knockout. Therefore, if CENP-A is

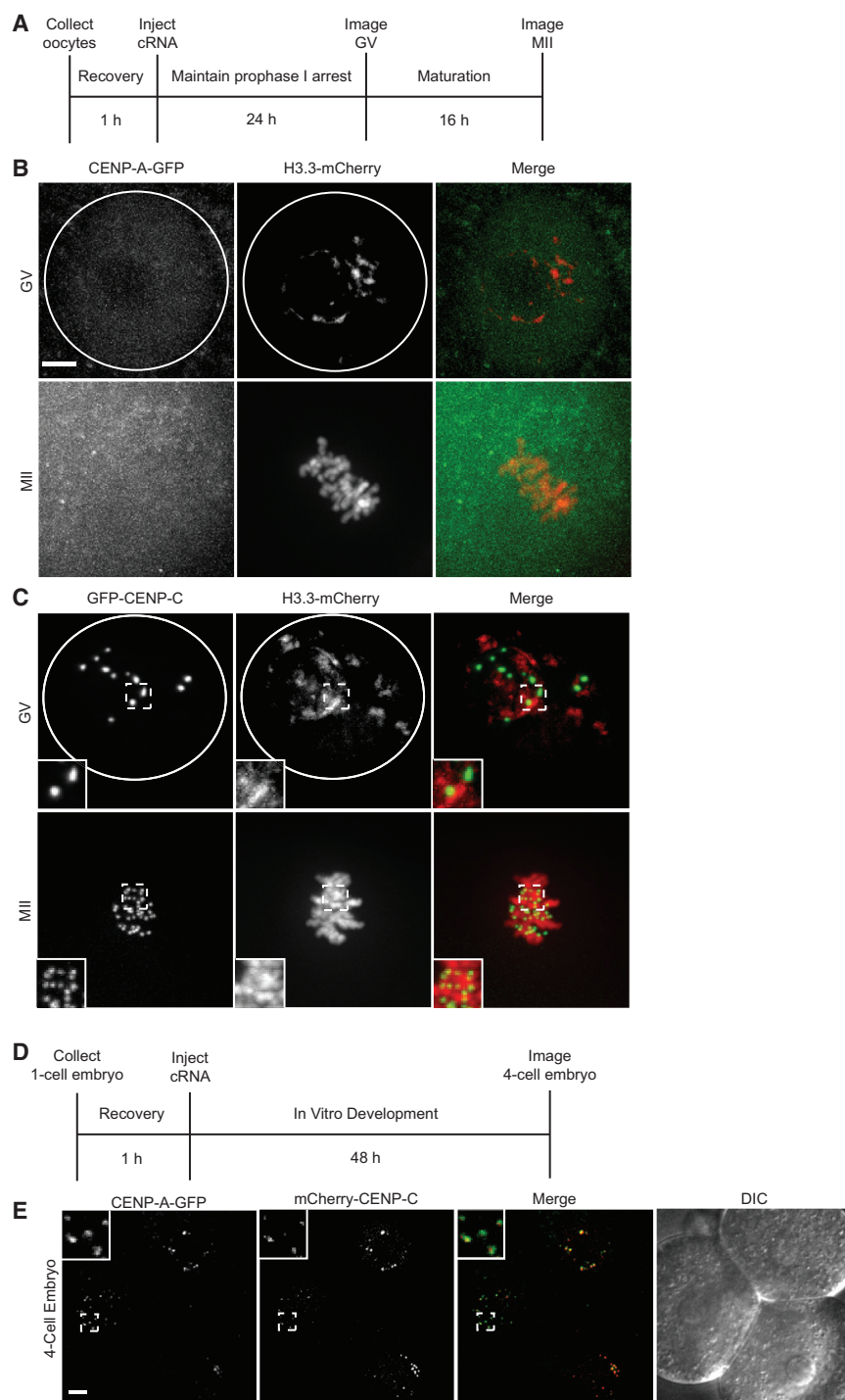


Figure 1. Absence of CENP-A Chromatin Assembly in Full-Grown Oocytes or MII Eggs

(A–C) Oocytes were injected with cRNA for CENP-A-GFP and H3.3-mCherry or GFP-CENP-C and H3.3-mCherry and imaged live in germinal vesicle-intact (GV) or metaphase II (MII) stages as shown in the schematic (A). Representative images show CENP-A-GFP and H3.3-mCherry (B) or GFP-CENP-C and H3.3-mCherry (C) at GV or MII stages ($n \geq 10$ in each case). Images are maximal intensity projections of confocal z series; insets show 1.6 \times magnification of centromeres labeled with GFP-CENP-C. White circle represents nuclear envelope.

(D) One-cell-stage embryos were injected with cRNA for mCherry-CENP-C and CENP-A-GFP and imaged live at the four-cell stage, as shown in the schematic.

(E) Representative images show an optical section of three blastomeres ($n = 27$); insets show 3 \times magnification of CENP-A-GFP and mCherry-CENP-C localized at centromeres. The scale bars represent 5 μ m.

See also Figure S1.

in centromeric CENP-A protein levels over time (Figure 2B, model 3). Because a pool of long-lived CENP-A protein in the oocyte would confound interpretation of our gene-deletion experiment, we measured total CENP-A protein in oocytes as compared to cycling NIH 3T3 cells. We found that 500 oocytes (4n) have approximately the same amount of CENP-A protein as $\sim 1,000$ NIH 3T3 cells (with each cell between 3 and 6n, because they are near tetraploid and from an asynchronous cycling population; Figure S2A). These data exclude the possibility that a large pool of excess CENP-A protein exists in oocytes to replenish CENP-A at centromeres in the absence of new synthesis.

To conditionally knock out *Cenpa*, we generated a mouse carrying a *Cenpa* allele in which exons 2–5 are flanked by *loxP* sites (Figure 2C) and used cre-recombinase driven by the *Gdf9* promoter to inactivate *Cenpa* in oocytes in resting primordial follicles [12]. Multiple lines of evidence indicate that the *Gdf9* promoter is active in these oocytes at an early

stage. First, our microarray data show a high level of *Gdf9* mRNA in isolated oocytes from resting primordial follicles collected 2 days after birth (day 2) [13]. Second, in an experimental system in which β -gal expression depends on *Gdf9-Cre* expression, primordial follicles stain positive at day 12 [12]. Because the first wave of follicle development begins at day 5, the β -gal-positive oocytes in these primordial follicles must be in the resting state. Third, *Gdf9-Cre*-mediated deletion of the

simply lost at oocyte centromeres, as in the worm (Figure 2B, model 1) [5, 6], then CENP-A should become undetectable in both knockout and control oocytes. Alternatively, if centromere inheritance depends on meiotic assembly of nascent CENP-A nucleosomes, then centromeric CENP-A levels should decrease in the knockout oocytes over time (Figure 2B, model 2). Finally, if centromere inheritance relies on the stable retention of preassembled CENP-A nucleosomes, then we expect little change

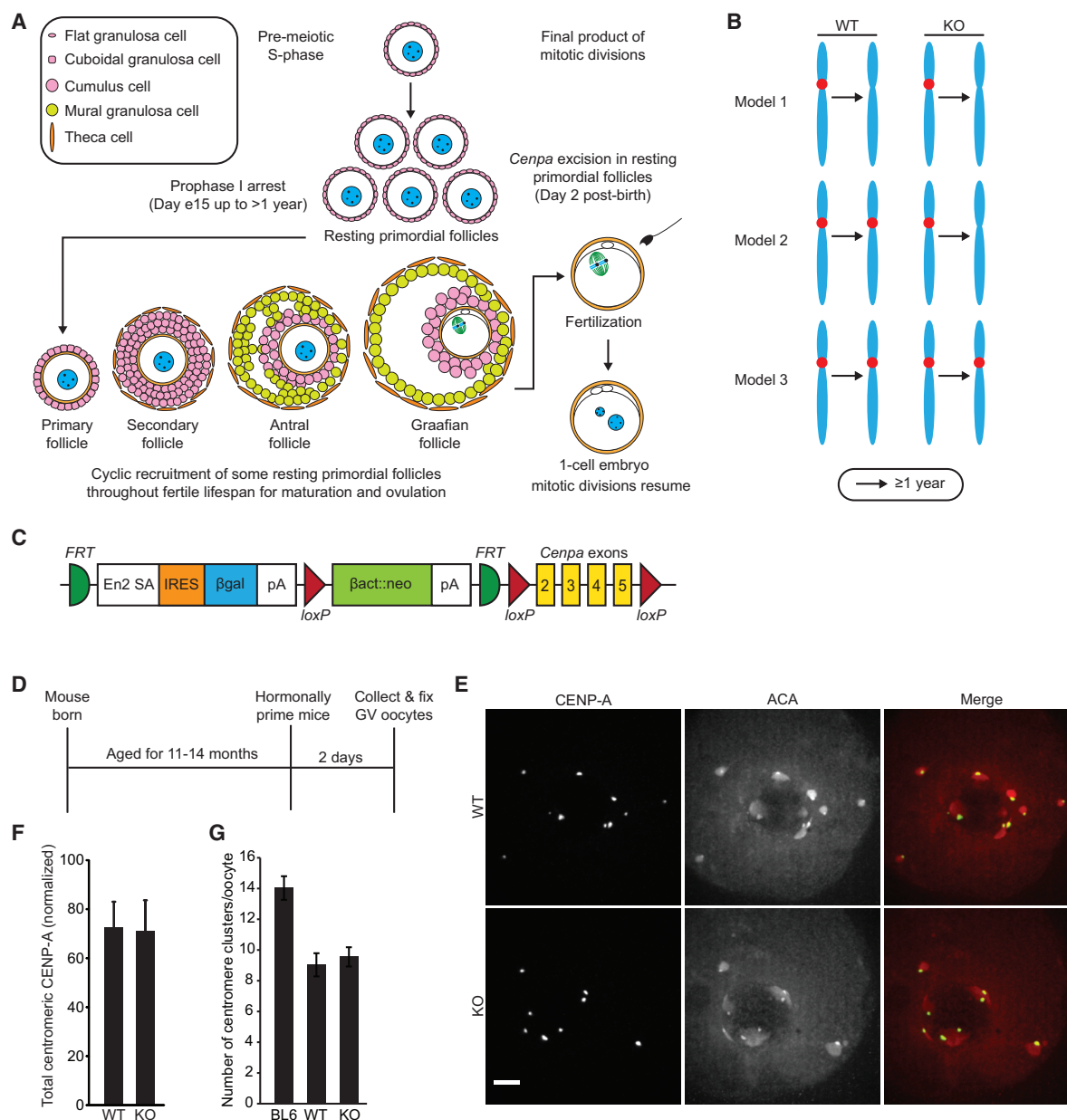


Figure 2. CENP-A Nucleosomes Are Stably Retained at Oocyte Centromeres for >1 Year

(A) Oocytes arrest in prophase I in resting primordial follicles, which are cyclically recruited starting 5 days after birth to begin growth toward a Graafian follicle, the final stage before ovulation. Primordial follicles can remain in the resting phase for a period lasting >1 year before they are recruited for maturation and ovulation. After fertilization, the maternal and paternal pronuclei enter G1 phase and begin mitotic cell cycles. Cre expression driven by the *Gdf9* promoter excises *Cenpa* in resting primordial follicles 2 days after birth.

(B) Three models for centromere inheritance in the mammalian oocyte. See [Results and Discussion](#) for details.

(C) Schematic of *Cenpa* conditional knockout gene locus. The neomycin selection cassette used for selection of embryonic stem cells (ESCs) is flanked by FRT sites. *Cenpa* protein coding exons 2–5 are flanked by loxP sites.

(D–F) Oocytes were collected from 11- to 14.5-month-old WT and KO mice or from young C57BL/6J (BL6) controls, and CENP-A levels were analyzed by immunofluorescence, with anti-centromere antibodies (ACA) to co-label centromeres (schematic; D). Images (E) of oocytes with intact GVs are maximal intensity projections of confocal z series; scale bar 5 μ m. Total centromeric CENP-A staining was quantified for each oocyte (n = 64 oocytes from four WT mice; n = 85 oocytes from five KO mice) and normalized to young C57BL/6J controls (n = 155 oocytes; eight mice) for each experiment. Normalized values were averaged over multiple experiments (F; error bars, SD).

(G) The number of centromere clusters was counted in each oocyte and averaged over each group (error bars, SD).

See also [Figures S2–S4](#) and [Table S1](#).

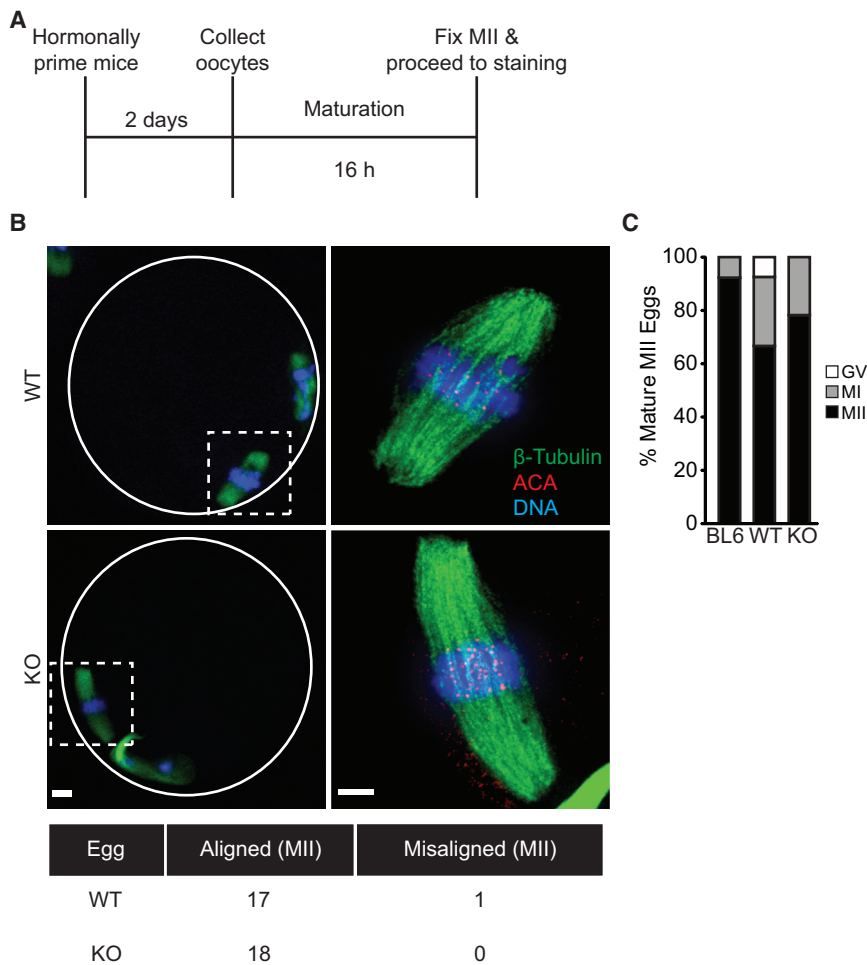


Figure 3. CENP-A Nucleosomes Assembled Early in Prophase I Support Normal Meiotic Centromere Function

(A) Oocytes were collected from 12-month-old WT and KO females or young C57BL/6J controls, matured in vitro to MII, and stained for β -tubulin, ACA to label centromeres, and DNA (schematic). (B) Representative images show MII eggs at 20 \times magnification (scale bar, 10 μ m); white circle represents zona pellucida; dashed squares are regions imaged at 100 \times (scale bar, 5 μ m). The table summarizes how many MII eggs from WT and KO females had chromosomes aligned at metaphase.

(C) The percent in each group that remained arrested with an intact GV, arrested at metaphase I, or progressed normally to MII was quantified ($n = 27$ oocytes from three WT mice, 23 oocytes from three KO mice, or 26 oocytes from two C57BL/6J [BL6] mice).

highly clustered in young oocytes and become even more clustered with age (Figure 2G). We measured total centromeric CENP-A fluorescence in each oocyte (Figures S3F–S3H) and normalized the signal to that in oocytes obtained from young C57BL/6J controls to compare biologically replicated experiments (Figure 2F). We find that aged KO and WT oocytes are indistinguishable, inconsistent with nascent CENP-A nucleosome assembly during the prolonged prophase I arrest (Figure 2B, model 2).

Similarly, cohesion is established in premeiotic S phase without detectable cohesin turnover during prophase I [17]. Further, both the WT and KO groups retain $\sim 70\%$ of the CENP-A protein seen in the young C57BL/6J group, showing that the majority of CENP-A present since early prophase I arrest in the primordial follicle is retained at the centromere for ~ 1 year. This retention is also inconsistent with a model where CENP-A is removed from centromeres in oocytes and then centromere identity is re-established in the early embryo, as in worms (Figure 2B, model 1) [5, 6]. These findings are, however, entirely consistent with a model (Figure 2B, model 3) where CENP-A nucleosomes assembled at the centromere before the prophase I arrest maintain centromere identity and function for the fertile lifespan of the female.

A broadly conserved feature of eukaryotic centromeres is the essential role of CENP-A nucleosomes in building a functional kinetochore [18–23]. To test whether the persistence of CENP-A nucleosomes at the centromere, rather than persistent expression, suffices for its essential function in the oocyte, we measured progression to MII and chromosome alignment on MII spindles (Figure 3A). Oocytes collected from 12-month-old KO and WT mice were similar in both assays, consistent with our finding that centromeric CENP-A levels are equivalent between the two groups (Figures 3B and 3C). Fewer oocytes from the 12-month groups matured to MII eggs compared to

Tsc1 or *Pten* genes, which are essential to maintain quiescence of primordial follicles, leads to activation of the entire pool of primordial follicles by day 23 [14, 15]. Finally, *Gdf9-Cre*-mediated expression of diphtheria toxin leads to complete loss of oocytes by 8 weeks of age [16]. We derived *Cenpa*^{fl/fl}; *Gdf9-Cre*⁻ (wild-type [WT]) or *Cenpa*^{fl/fl}; *Gdf9-Cre*⁺ (knockout [KO]) females in a C57BL/6J background (Figure S2B; Table S1). To confirm that the *Cenpa* gene locus is deleted in KO oocytes, we measured *Cenpa* mRNA levels by analyzing cDNA libraries created by reverse transcription of pooled mRNA from KO or WT oocytes (Figure S3A). We found that KO oocytes from young animals (14–19 weeks) contain only trace amounts of *Cenpa* mRNA (0.7% \pm 0.3%) relative to that present in WT oocytes (Figures S3B and S3C). Additionally, we detected measurable differences in *Cenpa* mRNA when cDNA from KO and WT oocytes was mixed in the following ratios: 100% KO; 90% KO/10% WT; and 95% KO/5% WT (Figures S3D and S3E). These data demonstrate that the *Cenpa* locus is efficiently excised in all KO oocytes and exclude the possibility of a stored pool of long-lived *Cenpa* mRNA.

Now positioned to test models for how centromere identity is maintained in mammalian oocytes, we measured centromeric CENP-A levels in the oocytes of WT and KO mice after aging them for 11–14.5 months (Figures 2D–2F). Centromeres are

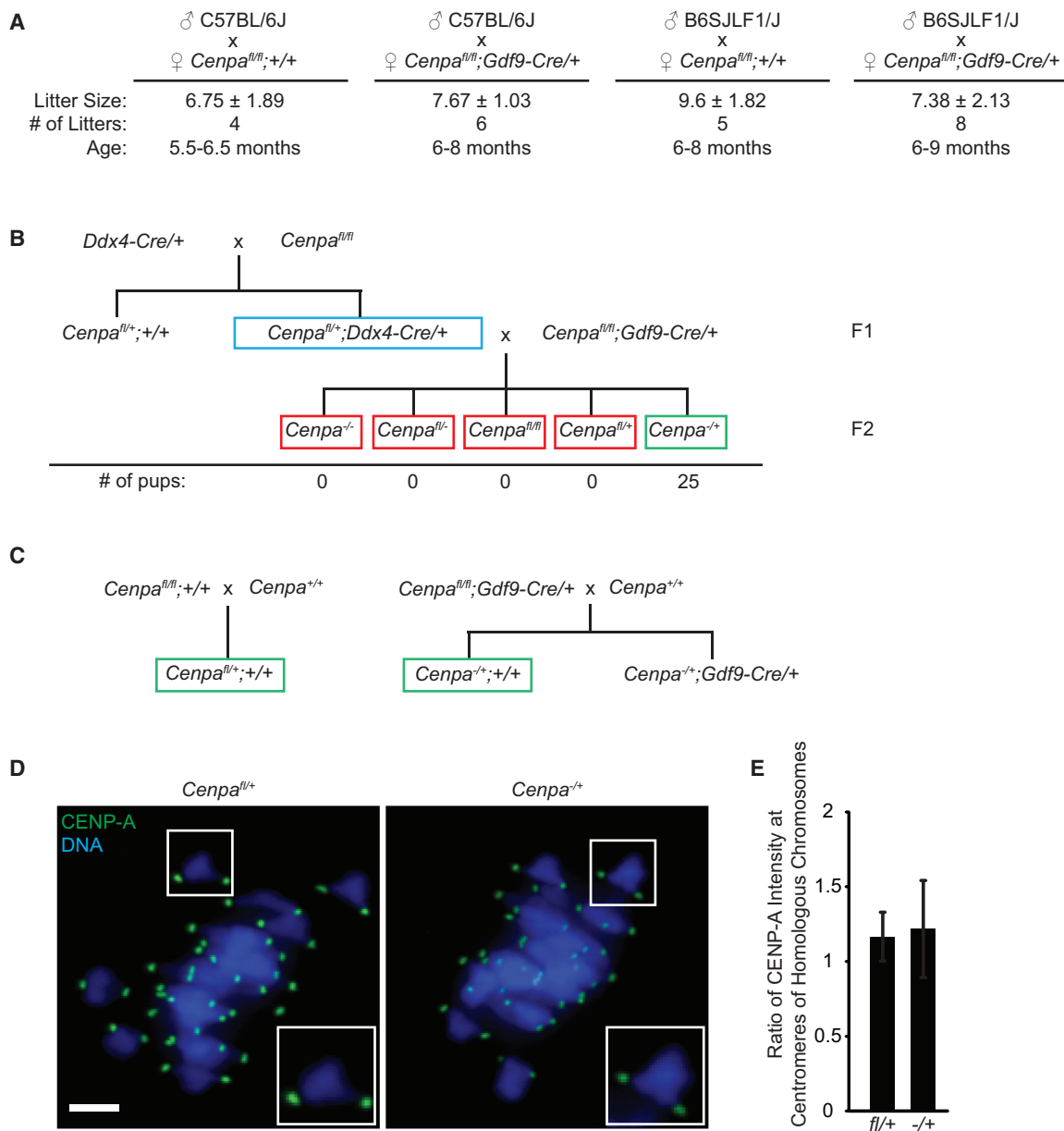


Figure 4. CENP-A Nucleosomes Assembled in Early Prophase I Support Normal Fertility and Transgenerational Centromere Inheritance

(A) WT (n = 5) and KO (n = 6) females were mated with either C57BL/6J or B6SJLF1/J males to determine fertility. Average litter size (±SD), number of litters, and age of the females are reported.

(B) *Cenpa^{fl/+};Ddx4-Cre/+* males (blue box) were mated to *Cenpa^{fl/m};Gdf9-Cre/+* females (n = 2 females; 3–6 months old). The red box represents offspring that are either predicted to die in utero (all *Cenpa^{-/-}*) or are predicted to be impossible to produce if there is full excision of the *Cenpa^{fl}* allele. The green box represents animals that are predicted to survive (all *Cenpa^{-/+}*) if all oocytes are indeed *Cenpa^{-/-}*. Surviving pups were genotyped and found to be exclusively *Cenpa^{-/+}* with no intact *Cenpa^{fl}* allele.

(C) Breeding scheme to generate F1 pups that are *Cenpa^{fl/+}* or *Cenpa^{-/+}*. Green boxes indicate mice used in experiment.

(D) Oocytes from F1 pups were fixed in metaphase I and stained for CENP-A and DNA.

(E) CENP-A intensity was measured at each centromere, and the ratio of brighter/dimmer intensity was calculated for each bivalent (n ≥ 238 bivalents for WT and KO). The error bars represent SD. The scale bar represents 5 μm.

young C57BL/6J controls, consistent with age-related meiotic defects [24–26].

As the ultimate test of centromere function after *Cenpa* deletion in oocytes, we measured fertility of WT and KO females. We used 5- to 9-month-old females, as older animals (WT or

KO) have prohibitively low fecundity and thus yield too few data points. We find that fertility is indistinguishable between WT and KO females, indicating that CENP-A nucleosomes assembled at centromeres in oocytes before the prophase I arrest provide full fertility months later (Figure 4A). Prior to receiving

CENP-A protein, message, and/or gene from the WT sperm, meiotic divisions in the oocyte would be completed with the CENP-A nucleosomes assembled 5–9 months earlier.

Our findings demonstrate that *Cenpa* deletion early in the prophase I arrest has no effect on oocyte maturation or female fertility. To independently verify that the *Cenpa* locus was deleted, we designed a cross in which survival of half of the offspring would depend on the presence of an intact maternal *Cenpa* allele in the egg (Figure 4B). We used the *Ddx4* promoter, which turns on in primordial germ cells, to excise a floxed *Cenpa* allele in spermatogonial stem cells [27]. Thus, *Cenpa*^{fl/+}; *Ddx4-Cre*/+ males produce mature sperm that are either *Cenpa*[−] or *Cenpa*⁺. If *Cenpa* is deleted in oocytes from KO females, all eggs produced will be *Cenpa*[−]. Crossing a KO female with a *Cenpa*^{fl/+}; *Ddx4-Cre*/+ male should produce embryos that are either *Cenpa*^{−/+} or *Cenpa*^{−/−}, depending on the sperm genotype. *Cenpa*^{−/+} animals are viable and fertile, but *Cenpa*^{−/−} is an embryonic lethal phenotype [20], so the surviving offspring from the cross should all be *Cenpa*^{−/+} and the litter sizes should be small. If *Cenpa* is not deleted in all oocytes, some offspring will be *Cenpa*^{fl/+} or *Cenpa*^{fl/−}. We find that pups generated from this cross are exclusively *Cenpa*^{−/+} (Figure 4B), confirming that the *Cenpa* locus is excised in all KO female oocytes. The viability of the *Cenpa*^{−/+} pups provides additional evidence that the CENP-A nucleosomes present at centromeres in KO oocytes are sufficient to produce a viable MII egg and support early embryogenesis.

To determine whether CENP-A nucleosomes present early in the prophase I arrest are sufficient for transgenerational inheritance of centromere identity, we examined the offspring of KO females crossed to normal C57BL/6J males. Defects in centromere inheritance between the maternally and paternally inherited chromosomes would be most apparent in metaphase I of the offspring, when the homologous chromosomes are paired. We crossed WT or KO females with C57BL/6J males, collected oocytes from F1 pups that were either *Cenpa*^{fl/+} or *Cenpa*^{−/+} (Figure 4C), and matured them to metaphase I in vitro. On each bivalent, we measured CENP-A levels on each side (i.e., at the centromeres of the homologous chromosomes) by immunofluorescence. If centromere inheritance was partially impaired, we would expect asymmetric CENP-A staining on each bivalent, which would imply that the maternal centromeres inherited less CENP-A. We find that it is symmetric, however, and the ratio of CENP-A staining within each bivalent is close to one for both genotypes (Figures 4D and 4E). Thus, faithful transgenerational centromere inheritance is maintained by the stable retention of CENP-A nucleosomes through the extended prophase I arrest of the oocyte.

Conclusions

Taken together, our results provide clear genetic evidence that stable retention of CENP-A nucleosomes underlies centromere inheritance through the mammalian female germline. CENP-A protein incorporated into centromeric chromatin before the prophase I arrest, most likely in the G1 phase preceding meiotic entry, displays spectacular longevity and is sufficient to provide essential centromere function for >1 year. This mechanism of centromere inheritance represents a new paradigm when compared to what has been described in other organisms

[5–7]. Our results stand in stark contrast to recent reports in which deposition of another histone H3 variant, H3.3, during prophase I is required for normal chromatin structure and gene expression and oocyte survival [11, 28]. Thus, centromeric chromatin remains extremely stable while nucleosome assembly in bulk chromatin is ongoing and essential during the extended prophase I arrest. Pericentromeric heterochromatin (e.g., immunostaining for HP1β and H3K9me3; Figure S4) is similar between oocytes and NIH 3T3 cells, so we prefer the notion that the stability we observe in the oocyte is a property conferred by CENP-A nucleosomes [29] themselves and/or other components of the centromere [2, 30]. In addition to the important implications for understanding how centromere identity is transmitted transgenerationally in mammals, our findings also advance what we know about the functions of long-lived proteins. Many of the most-remarkable examples are from metabolically inactive environments (e.g., crystallin and collagen) [31, 32] or of some nuclear components of non-dividing neurons [33–35]. Our findings extend the role of long-lived proteins to one that plays a central and essential role in the orchestration of chromosome segregation in the quintessential totipotent cell type. At the centromere, the remarkable stability of CENP-A nucleosomes through the fertile lifespan of the female cements the epigenetic information required to faithfully guide chromosome inheritance and transmit the chromosomal location of the centromere to offspring.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, one table, and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2016.02.061>.

AUTHOR CONTRIBUTIONS

E.M.S., P.S., R.M.S., M.A.L., and B.E.B. designed the experiments. E.M.S. and P.S. conducted the experiments. E.M.S., P.S., R.M.S., M.A.L., and B.E.B. analyzed the data. E.M.S., M.A.L., and B.E.B. wrote the manuscript.

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