Figure S1

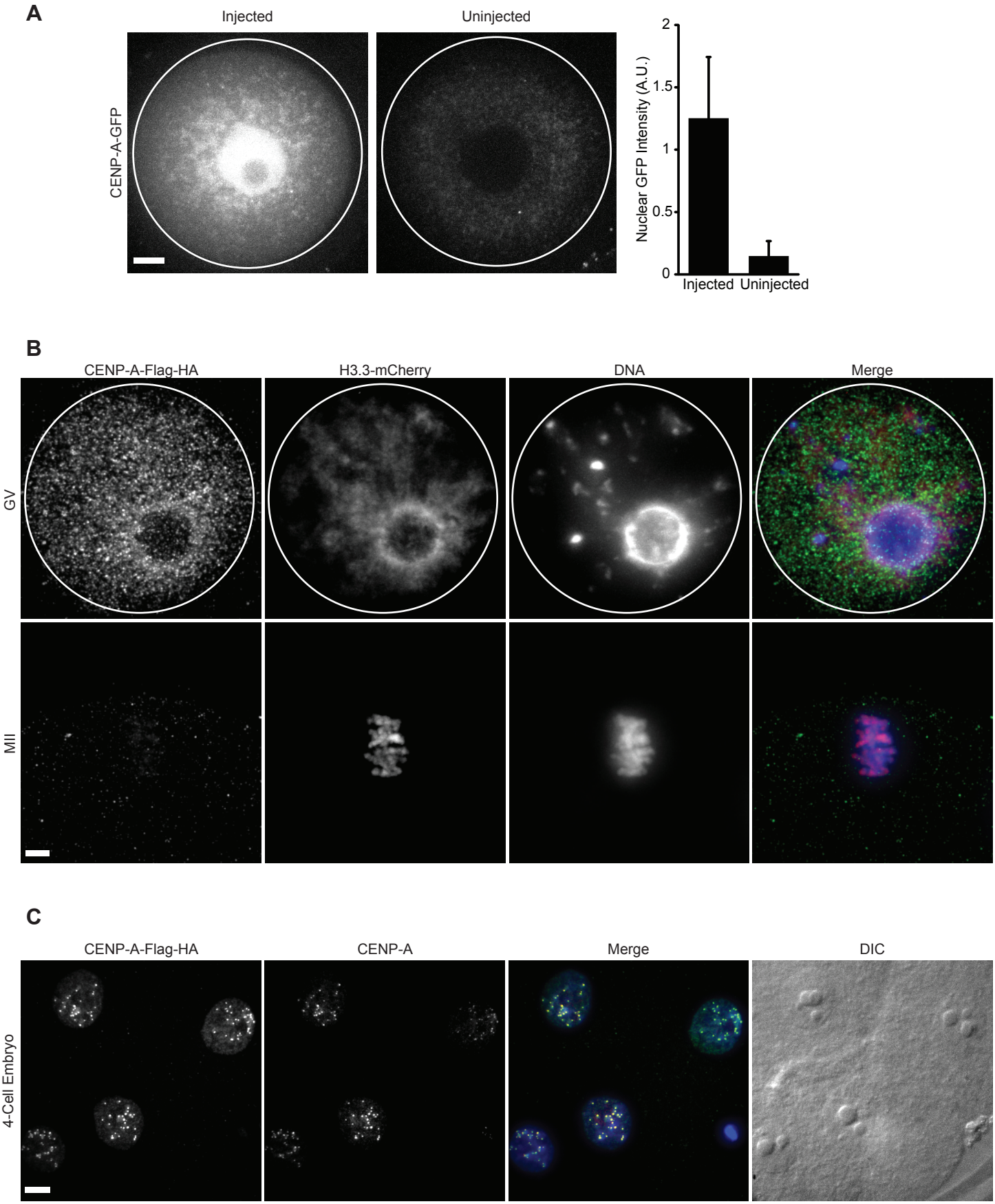
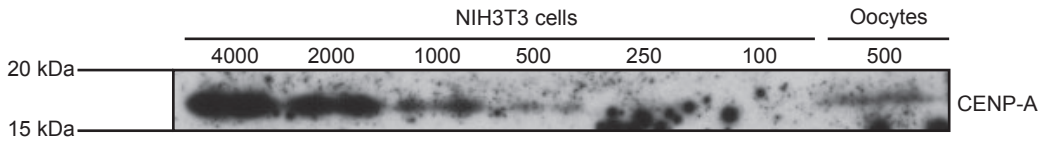

A
Injected
Uninjected
CENP-A-GFP
Nuclear GFP Intensity (A.U.)
2
1.5
1
0.5
0
Injected
Uninjected
B
CENP-A-Flag-HA
H3.3-mCherry
DNA
Merge
GV
MII
C
CENP-A-Flag-HA
CENP-A
Merge
DIC
4-Cell Embryo

Figure S2

A


NIH3T3 cells
Oocytes
4000
2000
1000
500
250
100
500
20 kDa
15 kDa
CENP-A

B

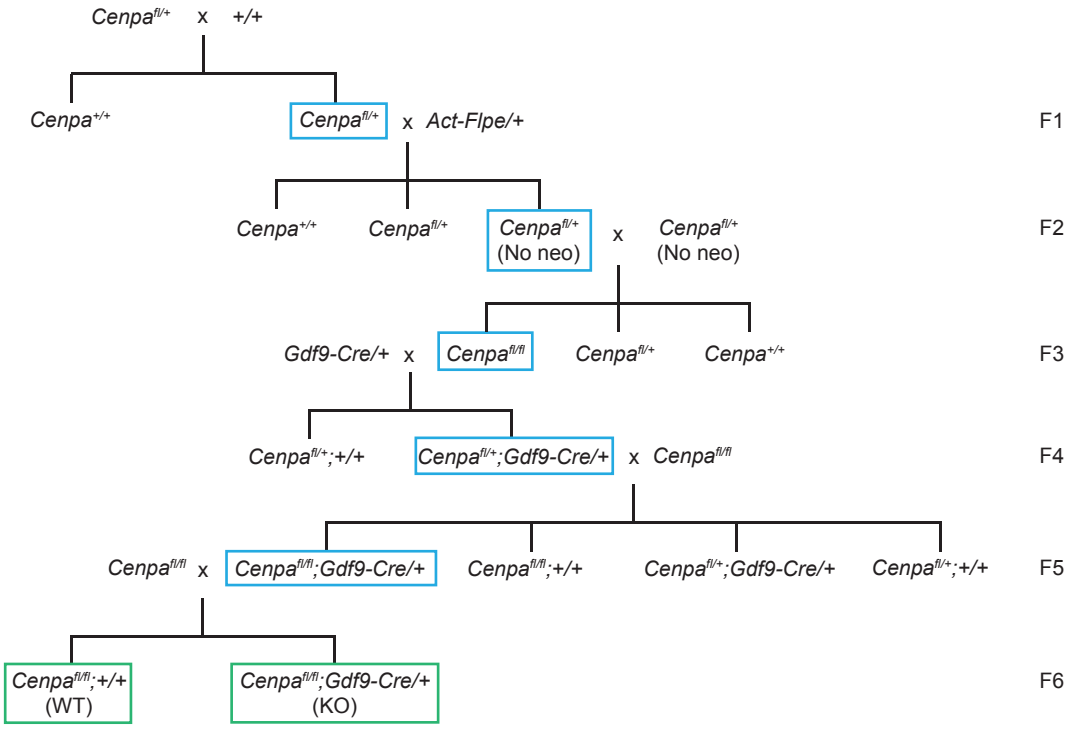

Cenpa fl/+ x +/+
Cenpa +/+
Cenpa fl/+ x Act-Flpe/+
F1
Cenpa +/+
Cenpa fl/+
Cenpa fl/+ (No neo) x Cenpa fl/+ (No neo)
F2
Gdf9-Cre/+ x Cenpa fl/fl
Cenpa fl/+
Cenpa +/+
F3
Cenpa fl/+;+/+
Cenpa fl/+;Gdf9-Cre/+ x Cenpa fl/fl
F4
Cenpa fl/fl x Cenpa fl/fl;Gdf9-Cre/+
Cenpa fl/fl;+/+
Cenpa fl/+;Gdf9-Cre/+
Cenpa fl/+;+/+
F5
Cenpa fl/fl;+/+ (WT)
Cenpa fl/fl;Gdf9-Cre/+ (KO)
F6

Figure S3

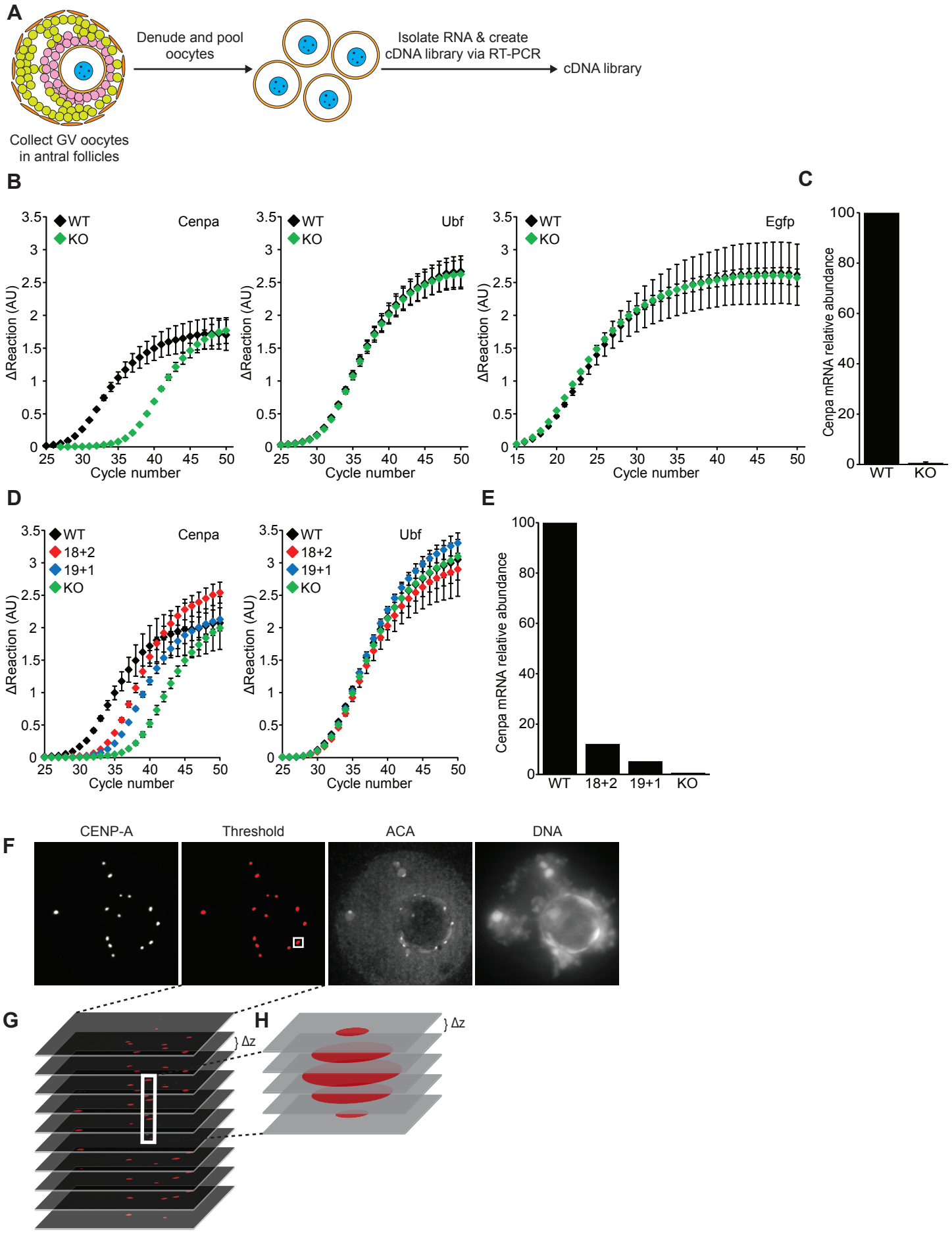
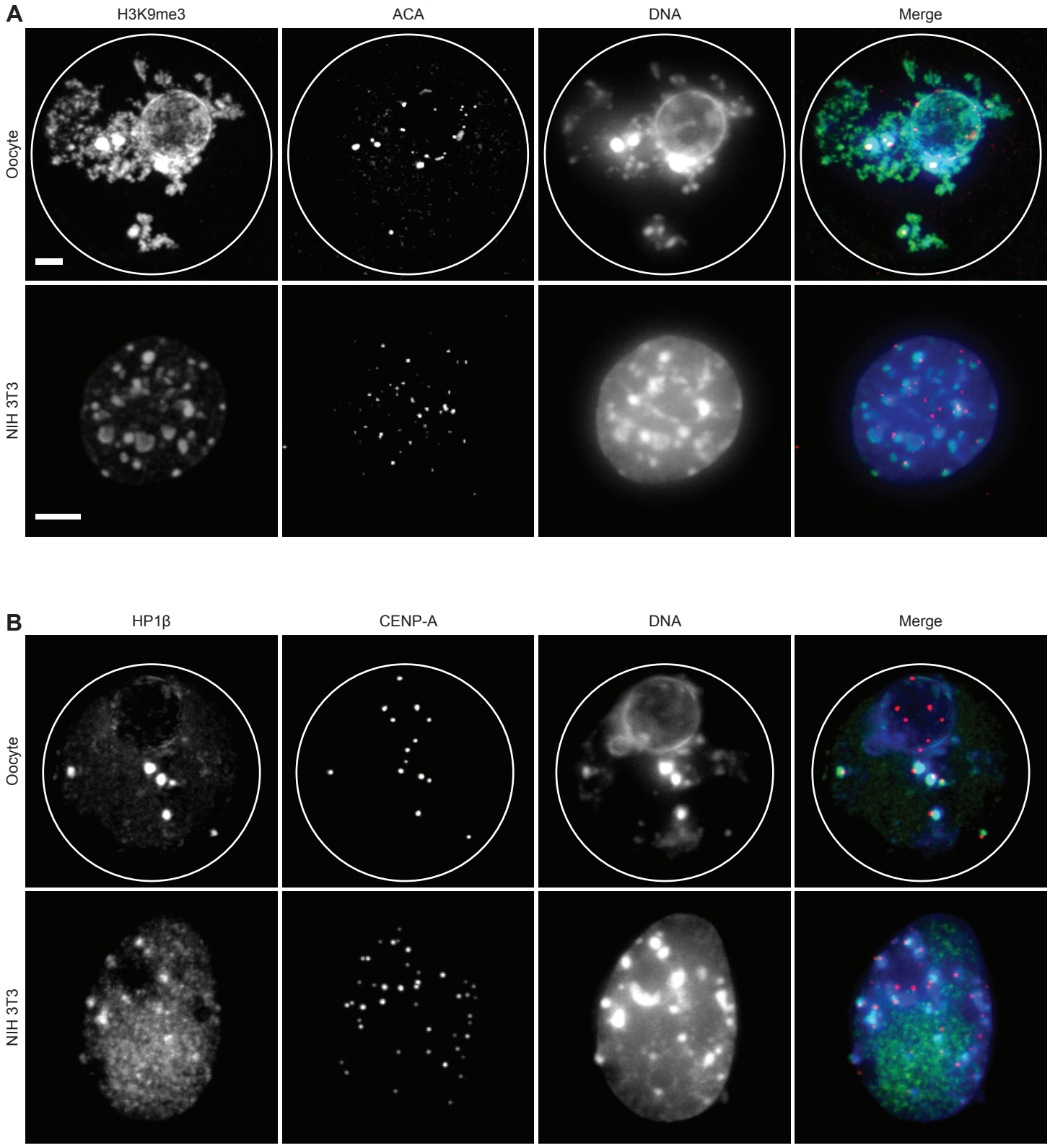

A
Denude and pool oocytes
Isolate RNA & create cDNA library via RT-PCR
cDNA library
Collect GV oocytes in antral follicles
B
WT
KO
Cenpa
Ubf
Egfp
ΔReaction (AU)
Cycle number
C
Cenpa mRNA relative abundance
WT
KO
D
18+2
19+1
E
F
CENP-A
Threshold
ACA
DNA
G
H
Δz

Figure S4


A
H3K9me3
ACA
DNA
Merge
Oocyte
NIH 3T3
B
HP1β
CENP-A
DNA
Merge
Oocyte
NIH 3T3

Supplemental Figure Legends:

Figure S1, Related to Figure 1: Oocytes injected with CENP-A-GFP cRNA have increased GFP fluorescence; absence of CENP-A loading in oocytes injected with CENP-A-Flag-HA cRNA.

(**A**) GV oocytes injected with cRNA encoding CENP-A-GFP display an increased GFP fluorescence compared to uninjected controls, though no CENP-A-GFP localizes to centromeres (n=20 for both injected and uninjected oocytes). White circle represents zona pellucida. Error bars s.d. (**B**) Oocytes were injected with cRNA for CENP-A-Flag-HA and H3.3-mCherry and fixed and stained with HA antibodies at either the germinal vesicle-intact (GV) or metaphase II (MII) stages. Representative images show CENP-A-Flag-HA and H3.3-mCherry. Images are maximal intensity projections of confocal z-series. White circle represents nuclear envelope. (**C**) 1-cell stage embryos were injected with CENP-A-Flag-HA and fixed and stained with CENP-A and HA antibodies at the 4-cell stage**.** Scale bars 10 μm.

Figure S2, Related to Figure 2: Oocytes do not contain a pool of excess CENP-A protein; *Cenpa* Conditional KO Breeding Scheme.

(**A**) Immunoblot of total CENP-A protein from whole cell lysates from an unsynchronized population of cycling NIH 3T3 cells or from CF-1 oocytes. Lysates from the indicated numbers of NIH 3T3 cells, from 100 to 4,000, are compared to 500 oocytes. (**B**) Breeding scheme for generation of WT and KO animals used in experiments (green boxes). The neomycin cassette was removed in the F1 cross with mice homozygous for Flp-recombinase under the control of the human β-actin promoter. Blue boxes indicate mice kept at each stage and used in further crosses.

Figure S3, Related to Figure 2: *Cenpa* KO oocytes contain only trace amounts of *Cenpa* mRNA; CENP-A immunofluorescence quantification.

(**A**) Experimental schematic of cDNA library preparation. (**B**) Representative Real-Time qPCR data comparing relative abundance of *Cenpa* mRNA between WT and KO oocytes. 1 oocyte equivalent was loaded per well. *Ubf* was used as an endogenous control to normalize the total amount of cDNA recovered from WT and KO oocytes. *Egfp* was used as an exogenous control to normalize the total amount of cDNA recovered by reverse transcription of mRNA isolated from WT and KO oocytes. Error bars, s.d. (**C**) *Cenpa* mRNA relative abundance between WT and KO oocytes, using the comparative Ct method; error bars, s.d. (**D**) Representative real-time qPCR data comparing relative abundance of *Cenpa* mRNA between cDNA mixes prepared from 100% WT cDNA, 90% KO cDNA mixed with 10% WT cDNA (18+2), 95% KO cDNA mixed with 5% WT cDNA (19+1), or 100% KO cDNA. *Ubf* was used as an endogenous control. Error bars represent s.d. (**E**) *Cenpa* mRNA relative abundance between WT, 18+2, 19+1 and KO cDNA libraries, using the comparative Ct method; error bars, s.d. (**F**) Maximal intensity projections of confocal z-series showing CENP-A staining, CENP-A thresholding, ACA, and DAPI staining from a representative GV oocyte used in the quantification shown in Fig. 2F (**G**) Individual z-planes showing centromeres in the white box in (A) in three dimensions. Total centromeric CENP-A intensity is quantified within the thresholded areas, integrated in three dimensions for each particle. (**H**) Schematic of a single CENP-A particle. Integrated intensity is determined by multiplying the thresholded volume by the mean pixel intensity.

Figure S4, Related to Figure 2: Pericentromeric chromatin marks localize similarly in NIH 3T3 cells and GV oocytes.

(**A**) H3K9me3 and (**B**) HP1β staining in GV oocytes and NIH 3T3 cells shows foci which colocalize with heterochromatin and centromeres. White circle represents nuclear envelope. Scale bar 5 μm. Thus, there is no evidence that differences between oocytes and somatic cells in heterochromatin at pericentromeres explains any aspect of CENP-A retention. There could

certainly be a role for centromere components binding to CENP-A nucleosomes or the intrinsic properties of CENP-A nucleosomes themselves. Scale bar 5 μm.

Table S1, Related to Figure 2: Genotyping primers and thermocycler programs.

Cenpa Genotyping
Fwd Primer: 5'-GCAGAGCCACAGCTCCAGAGCA-3'
Rev Primer 1: 5'-CAGACTGCAGTTCCATCCCTACAGA-3'
Rev Primer 2: 5'-TGAACTGATGGCGAGCTCAGACC-3'

Band Sizes:

Cenpa Floxed Allele: 490 bp
Cenpa Wildtype Allele: 382 bp
Cenpa Recombined Allele: 285 bp

Initial Denaturation: 95ºC for 5 min
Denaturation: 95ºC for 30 sec
Annealing: 55ºC for 30 sec
Elongation: 72ºC for 2 min
30 cycles
Final Elongation: 72ºC for 5 min

Gdf9-Cre Genotyping
Fwd Primer: 5'-TCTGATGAAGTCAGGAAGAACC-3'
Rev Primer: 5'-GAGATGTCCTTCACTCTGATTC-3'

Band Sizes:

Gdf9-Cre: 499 bp

Initial Denaturation: 94ºC for 3 min
Denaturation: 94ºC for 30 sec
Annealing: 58ºC for 2 min
Elongation: 72ºC for 2 min
30 cycles
Final Elongation: 72ºC for 5 min

Flp Genotyping
Fwd Primer: 5'-CACTGATATTGTAAGTAGTTTGC-3'
Rev Primer: 5'-CTAGTGCGAAGTAGTGATCAGG-3'

Band Sizes:

Act-Flpe: 725 bp

Initial Denaturation: 94ºC for 3 min
Denaturation: 94ºC for 30 sec
Annealing: 58ºC for 1 min
Elongation: 72ºC for 1 min
35 cycles
Final Elongation: 72ºC for 2 min

Ddx4-Cre Genotyping
Fwd Primer: 5'-CACGTGCAGCCGTTTAAGCCGCGT-3'
Rev Primer: 5'-TTCCCATTCTAAACAACACCCTGAA-3'

Band Sizes:

Ddx4-Cre: 240 bp

Initial Denaturation: 94ºC for 3 min
Denaturation: 94ºC for 30 sec
Annealing: 67ºC for 1 min
Elongation: 72ºC for 1 min
35 cycles
Final Elongation: 72ºC for 2 min

Supplemental Experimental Procedures

Oocyte/Embryo collection, meiotic maturation, and culture

Mice were primed by intraperitoneal injection of 5 IU of equine chorionic gonadotropin (eCG) 48 h before oocyte collection. Full-grown, germinal vesicle (GV)-intact cumulus-enclosed oocytes were collected as previously described [S1] and denuded. The collection medium was bicarbonate-free minimal essential medium (Earle's salt) supplemented with polyvinylpyrrolidone (3 mg/mL) and 25 mM HEPES, pH 7.3 (MEM/PVP). Germinal vesicle breakdown was inhibited by including 2.5 μM milrinone [S2]. For microinjection experiments, oocytes were transferred to CZB medium [S3] containing 2.5 μM milrinone and cultured in an atmosphere of 5% CO_2 in air at 37°C. To assess oocyte maturation in vitro, oocytes were transferred to milrinone-free CZB medium and cultured for 16 h in 5% CO_2 in air at 37°C. For microinjection of 1-cell stage embryos, mice were consecutively injected with 5 IU of equine chorionic gonadotropic (eCG) and 5 IU of human chorionic gonadotropin (hCG) 48 h apart, then mated with B6D2F1/J males. 20 h after mating, 1-cell stage embryos were collected from oviducts in MEM/PVP with 3 mg/mL hyaluronidase, denuded, and cultured in KSOM [S4,S5] in an atmosphere of 5% CO_2 in air at 37°C before and after microinjection.

Microinjection

GV oocytes were microinjected with ~5 pL of cRNAs in MEM/PVP containing 2.5 μM milrinone as previously described [S6]. 1-cell stage embryos were injected with ~5 pL of cRNAs in MEM/PVP. cRNAs used for oocyte injections were CENP-A-GFP (600 ng/μl), CENP-A-Flag-HA (60ng/ul), H3.3-mCherry (300 ng/μL), 2xGFP-CENP-C (480 ng/μl) with two tandem copies of GFP. cRNAs used for embryo injections were CENP-A-GFP (600 ng/μl), CENP-A-Flag-HA (60ng/ul), and 3xmCherry-CENP-C cRNA (500 ng/ul) with three tandem copies of mCherry.

Tissue Culture

NIH 3T3 cells were cultured in growth medium (Dulbecco's Modified Eagle's Medium supplemented with 10% Fetal Calf Serum and penicillin-streptomycin) at 37ºC in a humidified atmosphere with 5% CO_2.

Generation of *Cenpa* Conditional KO Mice

ES cells with exons 2-5 of *Cenpa* flanked by loxP sites and an FRT-flanked neomycin selection cassette between the 5' and 3'arms (Figure 3A) (EuMMCR, $Cenpa^{tm1a(EUCOMM)Wtsi}$, Clone ID: EPD0445_6_E07, Cell Type: C57BL/6M) were injected into BALB/c blastocysts and transferred to pseudopregnant mothers to generate two chimeric founders (90% and 1-5% chimerism), which were germline transmitters of the mutant allele. The neomycin selection marker was removed with the FLP recombinase, by crossing the Neo::$Cenpa^{fl/+}$ mice with ACTB::Flpe mice (Stock #: 005703, The Jackson Laboratory). The resulting $Cenpa^{fl/fl}$ mice were mated with *Gdf9-Cre/+* (Stock #: 011062, The Jackson Laboratory) males, and the resulting $Cenpa^{fl/+}$*;Gdf9-Cre/+* males were mated with $Cenpa^{fl/fl}$ females to obtain $Cenpa^{fl/fl}$*;Gdf9-Cre/+* females. To obtain $Cenpa^{fl/+}$*;Ddx4-Cre/+* males, $Cenpa^{fl/fl}$ females were crossed to *Ddx4-Cre/+* males (Stock # 006954, The Jackson Laboratory). Mice were genotyped by PCR analysis of tail DNA extracted using the REDExtract-N-Amp Red Tissue PCR kit (Sigma-Aldrich) using primers and PCR programs listed in Table S1. We note that in a small fraction of animals generated from $Cenpa^{fl/fl}$*;Gdf9-Cre+* (♂) x $Cenpa^{fl/fl}$ (♀) were negative for *Gdf9-Cre* by our genotyping assay, but also genotyped as $Cenpa^{fl/-}$, indicating potentially aberrant Cre activity in the male. We avoided including any females with the $Cenpa^{fl/-}$ genotype in our analysis by genotyping with primers that detect $Cenpa^{fl}$, $Cenpa^{+}$, and $Cenpa^{-}$ alleles. All animal experiments were approved by the Institutional Animal Use and Care Committee of the University of Pennsylvania and were consistent with National Institutes of Health guidelines.

Indirect Immunofluorescence

Mouse oocytes were fixed in freshly prepared 2% paraformaldehyde in PBS (pH 7.4) for 20 min at room temperature, washed through 3 drops of blocking solution (PBS containing 0.1% BSA and 0.01% Tween-20), permeabilized in PBS with 0.1% Triton X-100 for 15 min, washed in blocking solution for 15 min, incubated 1 h with primary antibodies, washed three times in blocking solution for 15 min, incubated 1 h with secondary antibodies, washed three times for 15 min in blocking buffer, and mounted in Vectashield with DAPI (1.5 μg/mL, Sigma-Aldrich) to visualize DNA. In experiments where CENP-A staining was perfomed on metaphase oocytes, we incubated oocytes with λ-phosphatase (New England Biolabs, #P0753S) prepared according to manufacturer's instructions for 2 hours at 30ºC immediately after permeabilization/blocking before proceeding with antibody incubation. Primary antibodies were human ACA autoimmune serum (1:50, PerkinElmer), rabbit anti-CENP-A (1:200, Cell Signaling #2048S), and rabbit anti-β-tubulin (9F3) monoclonal conjugated to Alexa Fluor 488 (1:75; Cell Signaling #3623), H3K9me3 (1:500, Abcam ab8898) and HP1β (1:200, Millipore MAB3448), anti-HA.11 (1:200, Covance MMS-101p). Secondary antibodies were Alexa Fluor 594–conjugated goat anti–human, Alexa Fluor 488-donkey anti-rabbit, Alexa Fluor 488-donkey anti-mouse, and Alexa Fluor 594 donkey anti-rabbit (1:100), Invitrogen).

Image Acquisition

Confocal images were collected as z-stacks with 0.3 μm (microinjection and meiotic maturation) or 0.5 μm (GV oocyte immunofluorescence) intervals to visualize all chromatin (25-30 μm range) using a microscope (DMI4000 B; Leica) equipped with a 20x 0.7 NA dry-objective lens (meiotic maturation), a 63x 1.3 NA glycerol-immersion objective lens (microinjection), a 100x 1.4 NA oil-immersion lens (GV oocyte immunofluorescence and meiotic maturation), an xy piezo Z stage (Applied Scientific Instrumentation), a spinning disk confocal scanner (Yokogawa Corporation of America), an electron multiplier charge-coupled device camera (ImageEM C9100-13; Hamamatsu Photonics), and an LMM5 laser merge module with 488- and 593-nm

diode lasers (Spectral Applied Research) controlled by MetaMorph software (Molecular Devices). For live imaging, oocytes and embryos were cultured in drops of either CZB or KSOM in FluoroDish (FD35-100, World Precision Instruments, Inc.) covered by mineral oil to prevent evaporation. Temperature was maintained at 37ºC with 5% CO_2 using an environmental chamber (Incubator BL; PeCon GmbH).

Image Analysis

Image analysis was done in ImageJ. The total amount of centromeric CENP-A in each GV oocyte was quantified by first defining the z-slices containing centromeres, based on CENP-A and ACA staining. A threshold CENP-A intensity and minimum particle size were determined manually for each oocyte, using the Object Counter3D macro. The threshold intensity varied between oocytes, depending usually on how clustered the centromeres were. The volume and average intensity of each particle was recorded. Background fluorescence, estimated from ROIs adjacent to but not including centromeres, was subtracted, and the integrated intensity of each particle was calculated as average intensity x volume. The total centromeric CENP-A intensity in each oocyte is the sum of all of the particles. Co-localization of ACA and CENP-A staining was used to ensure that all particles were actually centromeres. Total centromeric CENP-A intensity was averaged over all oocytes from each mouse. In each experiment, age-matched WT and KO mice were analyzed in parallel with young (7-12 week) C57BL/6J controls. To compare mice from difference experiments, results were normalized to the young C57BL/6J controls within each experiment. These normalized values were then averaged together for WT and KO mice. To compare centromeres of homologous chromosomes at metaphase (Fig. 4E), the integrated intensity was calculated for each centromere as above and then a ratio was calculated as the brighter/dimmer centromere within each bivalent in all cases where homologous chromosomes could be unambiguously identified.

The GFP intensity in Fig. S1A was calculated by averaging 5 different nuclear GFP measurements of equivalent area and then subtracting the average of 5 different cytoplasmic GFP measurements for each oocyte.

Mating Assays

Females used in the fertility trials were housed with one male for 4 months, and the number of pups in each litter was recorded. Females that were mated to *Cenpa*$^{fl/+}$*;Ddx4-Cre/+* males were housed with one male for 6 months, and the number and *Cenpa* genotype of the pups in each litter were recorded.

Immunoblot

Samples derived from whole cell lysates were separated by SDS-PAGE and transferred to a nitrocellulose membrane for immunoblotting. Blots were probed using a rabbit anti-CENP-A antibody (1:200, Cell Signaling #2047S). Antibodies were detected using an ECL anti-rabbit IgG, horseradish peroxidase-conjugated secondary antibody (GE Healthcare, NA934V) at 1:2000 and Amersham ECL Select Western Blotting Detection Reagent (GE Healthcare, RPN2235).

mRNA quantitative RT-PCR

Total RNA was extracted from at least 21 full-grown oocytes using Trizol (Life Technologies), according to the manufacturer's protocol, except that 2 ng of *Egfp* RNA was added to the Trizol at the beginning of RNA isolation to serve as an exogenous normalization gene. cDNA was prepared by reverse transcription of total RNA with Superscript II and random hexamer primers. One oocyte equivalent of the resulting cDNA was amplified using TaqMan probes and the ABI Prism Sequence Detection System 7000 (Applied Biosystems). Two replicates were run for each real-time PCR reaction; a minus template served as control. Quantification was normalized to Egfp and endogenous Ubf within the log-linear phase of the amplification curve obtained for each probe/ primer using the comparative Ct method (ABI PRISM 7700 Sequence Detection

System, User Bulletin 2, Applied Biosystems, 1997). The TaqMan gene expression assays used were: Mm00483252_m1 (Cenpa), Mm00456972_m1 (Ubf). The TaqMan gene expression assay for Egfp was a custom order from ThermoFisher Scientific using the following primers: Forward: 5'- GCTACCCCGACCACATGAAG-3', Reverse: 5'- CGGGCATGGCGGACTT-3', Reporter Dye: FAM.

Supplemental References:

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S2. Wiersma, A., Hirsch, B., Tsafriri, A., Hanssen, R.G.J.M., Van De Kant, M., Kloosterboer, H.J., Conti, M., and Hsueh, A.J.W. (1998). Phosphodiesterase 3 inhibitors suppress oocyte maturation and consequent pregnancy without affecting ovulation and cyclicity in rodents. J. Clin. Invest., *102*, 532–7.

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S4. Erbach, G.T., Lawitts, J.A., Papaioannou, V.E., and Biggers, J.D. (1994). Differential growth of the mouse preimplantation embryo in chemically defined media. Biol. Reprod., *50*, 1027–33.

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S6. Kurasawa, S., Schultz, R.M., and Kopf, G.S. (1989). Egg-induced modifications of the zona pellucida of mouse eggs: effects of microinjected inositol 1,4,5-trisphosphate. Dev. Biol., *133*, 295–304.