Supporting Information

Wen et al. 10.1073/pnas.1406389111

SI Materials and Methods

Injection of siRNA and mRNA into MII Oocytes. The following sequences were used for siRNA and cDNA injection into oocytes. siRNA sequences for H3.3A (h3f3a) and H3.3B (h3f3b) were as follows:

h3f3a 1s, CGUUCAUUUGUGUGUGAAUUUUU; h3f3a 1as, AAAUUCACACACAAAUGAACGUU; h3f3a 2s, GCGAGAAAUUGCUCAGGACUUUU; h3f3a 2as, AAGUCCUGAGCAAUUUCUCGCUU; h3f3b 1s, UCUGAGAGAGAUCCGUCGUUAUU; h3f3b 1as, UAACGACGGAUCUCUCUCAGAUU; h3f3b 2s, GAAGCUGCCAUUCCAGAGAUUUU; h3f3b 2as, AAUCUCUGGAAUGGCAGCUUCUU.

cDNA sequence for H3.3-HA add-back construct was as follows: GCTCGTACTAAGCAGACCGCTCGCAAGTCCACCGGTGGCAAGGCCCCGCGTAAGCAGCTGGCTACCAAGGCCGCCCGCAAGAGCGCCCCGTCCACCGGCGGCGTGAAGAAGCCTCACCGCTACCGTCCCGGCACCGTGGCGCTGCGCGAGATCCGGCGCTACCAGAAGTCGACCGAGCTGCTGATCCGCAAGCTGCCGTTCCAGCGCCTGGTGCGCGAGATCGCGCAGGACTTCAAGACCGACCTGCGCTTCCAGAGCGCGGCCATCGGGGCTCTGCAGGAGGCCTCTGAGGCCTACCTTGTGGGTCTGTTTGAGGACACCAACCTGTGCGCCATCCACGCCAAGCGTGTCACCATCATGCCCAAGGACATCCAACTGGCCCGCCGCATTCGCGGGGAGAGGGCGGCGGCCGCTGGAGGATACCCCTACGACGTGCCCGACTACGCCTAG.

ES Cell and Mouse Embryonic Fibroblast Culture. ES cells were cultured on irradiated mouse embryonic fibroblasts (MEFs) in KO DMEM containing 15% FCS, leukemia inhibiting factor (LIF), penicillin/streptomycin, L-glutamine, β-mercaptoethanol, and nonessential amino acids. MEFs were cultured in DMEM supplemented with 10% FCS. MEFs were derived from embryonic day (E)13.5 chimera (H3.3KD1 mES cells or H3.3KO ES cells).

Derivation of ES Cell Lines from Fertilized Embryos and Nuclear Transfer Embryos. The ES cell derivation medium was composed of 75 mL knockout DMEM (cat. no. 10829–018; Gibco), 20 mL KO serum replacement (cat. no. 10828; Gibco), 1 mL penicillin/streptomycin (cat. no. TMS-AB-2C; Specialty Media), 1 mL L-glutamine (cat. no. TMS-001-C; Specialty Media), 1 mL nonessential amino acids (cat. no. TMS-001-C; Specialty Media), 1 mL nucleosides for ES cells (cat. no. ES-008-D; Specialty Media), 1 mL β-mercaptoethanol (cat. no. ES-007-E; Specialty Media), 250 μL PD98059 (cat. no. V1191; Promega), and 20 μL recombinant mouse LIF (cat. no. ESG1107; Chemicon).

Feeder cells of MEFs were obtained from E12.5 or E13.5 mouse embryos and inactivated with mitomycin C. Feeder layers were cultured in serum-supplemented medium overnight, and washed with 1× PBS solution to reduce serum just before plating embryos. Blastocysts of the nuclear transfer embryos were used for ES cell derivation. Zonae pellucidae of the blastocysts were removed by brief exposure to Tyrode saline solution acidified to pH 2.5. These denuded embryos were plated individually into a well of a 96-well plate covered with feeder cells, and cultured in the medium at 37 °C in 5% CO_2 in humidified air for 4–5 d. Cell clumps originated from the blastocysts were trypsinized in 20 μL of 0.025% trypsin and 0.75 mM EDTA (cat. no. SM-2004-C; Specialty Media) for 5 min, and 200 μL of ES medium was added to each well to stop the reaction. Cells were dispersed by pipetting up and down at least 20 times with a 200-μL pipetter, and the whole medium with cell suspension was transferred to another well with freshly seeded feeder cells in the 96-well plate. Cell colonies could be observed 2–3 d after the first trypsinization. Colony expansion of the putative ES cells proceeded from 48-well plates to six-well plates with feeder cells in ES medium, and then to gelatinized 25-cm^2 flasks for routine culture in regular ES culture medium, with 15% FCS and 1,000 IU/mL LIF. Cell aliquots were cryopreserved by using Cell Culture Freezing Medium (cat. no. ES-002-D; Specialty Media) and stored in liquid nitrogen.

Blastocyst Injection. Diploid or tetraploid blastocysts [94–98 h after human chorionic gonadotropin (hCG) injection] were placed in a drop of DMEM with 15% FCS under mineral oil. A flat-tip microinjection pipette was used for ES cell injections. Ten to fifteen injected blastocysts were transferred to each uterine horn of 2.5 d post coitum pseudopregnant B6D2F1 females. Embryos were collected at E13.5 for MEF derivation.

Quantitative RT-PCR. The following primers were used in this study: gapdh forward, ggttgtctcctgcgacttcaacagc, gapdh reverse, cgagttggatagggcctctcttgc; h3f3a forward, acaaaagccgctcgcaagag, h3f3a reverse, atttctcgcaccagacgctg; h3f3b forward, tggctctgagagagatccgtcgtt, h3f3b reverse, ggatgtctttgggcatgatggtgac; gfp forward, agccgctaccccgaccacat, gfp reverse, cggttcaccagggtgtcgcc. Hist1h3f forward, tggctcgtactaagcagacc reverse, aggttggtgtcctcaaacag; Hist2h3c2 forward, ggacttcaagacggacctg, reverse, gccaactggatgtccttg.

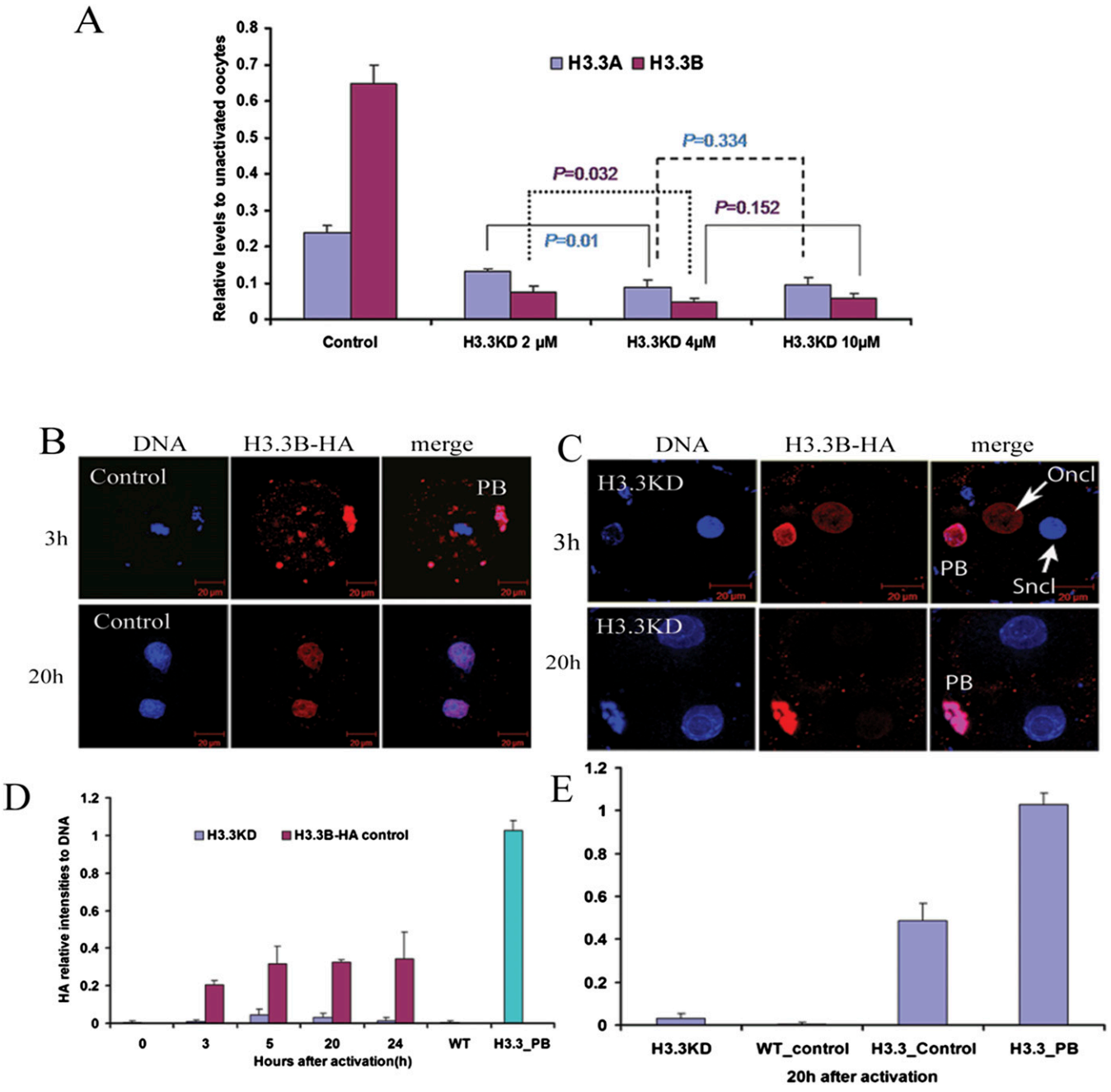

Fig. S1. (*A*) Knockdown efficiencies of siH3.3 for different injection concentrations. The knockdown efficiency for 4 μM siRNA against H3.3 (siH3.3) was not significantly different from that for 10 μM, whereas the maternal H3.3A and H3.3B were significantly decreased for 4 μM siH3.3 vs. 2 μM siH3.3. (*B*) Embryos produced by transfer of WT cumulus cell nuclei into enucleated H3.3B-HA oocytes, embryos were fixed at 3 h and 20 h after activation. Massive accumulation of maternal H3.3B-HA protein in the cytoplasm was observed 3 h after activation (arrow). PB, polar body (serves as H3.3B-HA positive control). Strong H3.3B-HA signals were also detected in the WT nuclei of somatic cell nuclear transfer (SCNT) embryos 20 h after activation (two-cell stage). (*C*) Embryos produced by transfer of WT cumulus nuclei into nucleated or enucleated oocytes after siH3.3 injection. PB and oocyte nucleus serve as positive control. H3.3B-HA protein accumulation was undetectable in the cytoplasm of the activated oocyte 3 h after activation in H3.3KD PA embryos. Maternal-derived H3.3B-HA signal was undetectable in the WT nuclei of H3.3KD two-cell stage embryos; PB serves as positive control and indicates the origin of the embryo. Oncl, oocyte nucleus; Sncl, somatic nucleus. (*D* and *E*) Time course of H3.3B-HA relative intensities in the WT nuclei of H3.3B-HA oocyte-SCNT embryos. H3.3B-HA signals were undetectable in the WT nuclei of H3.3KD SCNT embryos. H3.3B-HA control, SCNT embryos (H3.3B-HA oocytes and WT nuclei) without siH3.3 injection; H3.3B-HA_PBI, polar body of H3.3B-HA oocytes; H3.3KD, embryos constructed by transfer of WT cumulus nuclei into H3.3B-HA nucleated or enucleated oocytes after siH3.3 injection; WT_control, WT SCNT embryos.

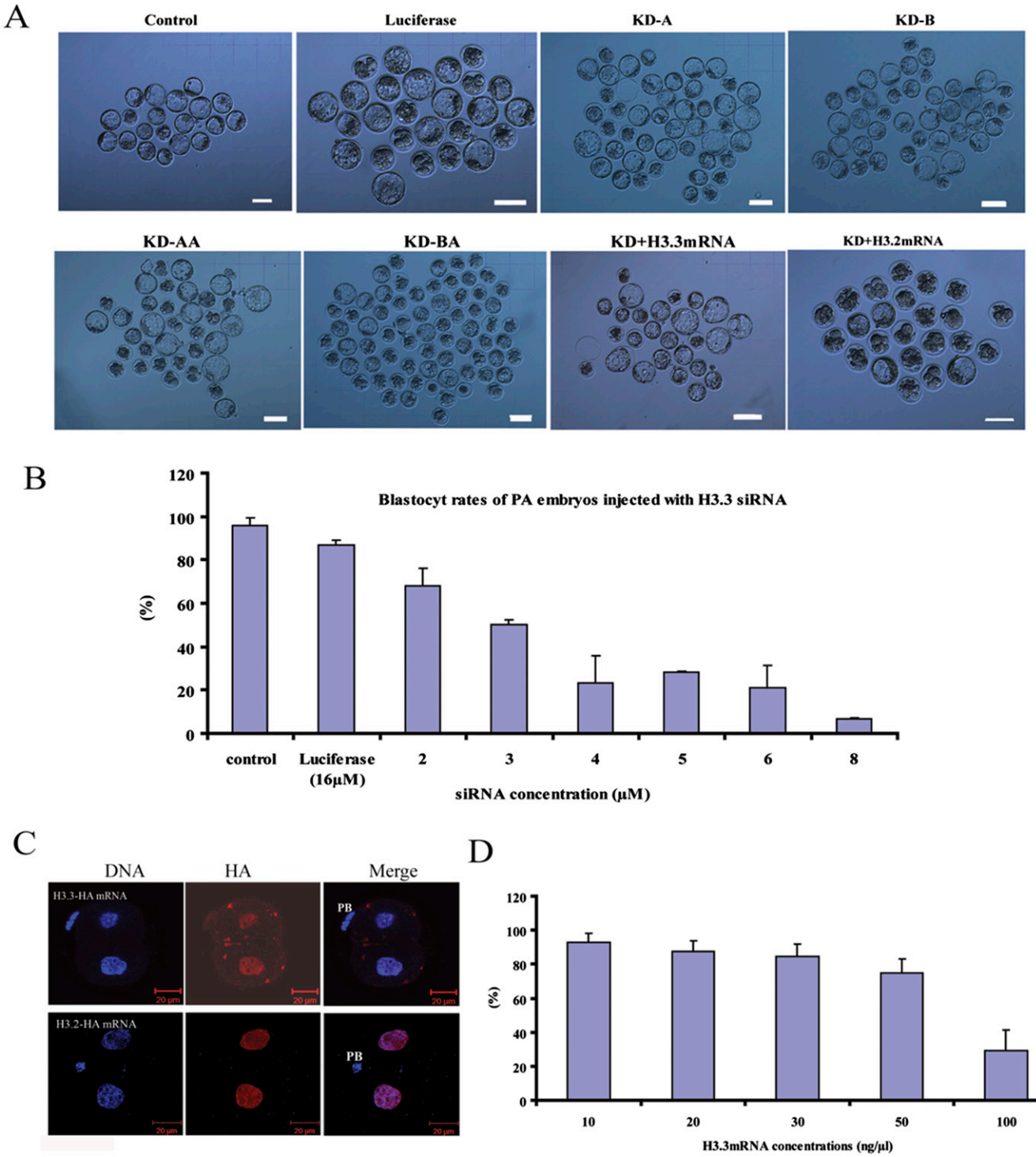

Fig. S2. (*A*) The blastocysts of PA embryos in Fig. 1*E*. (*B*) The developmental potentials of PA embryos decreased as increase of the siH3.3 concentration. (*C*) Injecting H3.3-HA mRNA or H3.2-HA mRNA into the WT oocytes before activation, H3.3-HA and H3.2-HA protein were detected in the nuclei of the two-cell stage embryos, whereas the PBs were H3.3-HA or H3.2-HA negative. (*D*) Determining the optimal injection concentrations of exogenous H3.3-HA mRNA into the oocytes. (Scale bar: 20 μm.)

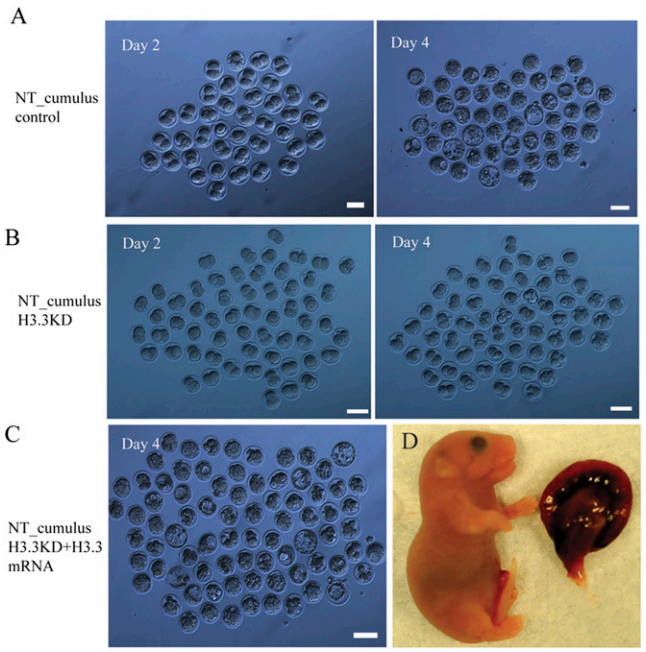

Fig. S3. (*A*) Cumulus (B6D2F1) SCNT embryos at day 2 and day 4. (*B*) Cumulus (B6D2F1) SCNT embryos using H3.3KD (4 μM) enucleated oocytes were normally completed the first cleavage, but most of them arrested at the two- to four-cell stage. (*C*) H3.3-addback (30 ng/mL H3.3-HA mRNA) cumulus nuclear transfer embryos at day 4 (4 μM siH3.3). (*D*) A cumulus cloned pup from 39 morula/blastocyst H3.3mRNA-rescued embryos; the pup was living after caesarean section, and no obvious defect was observed for this pup except the larger body weight (1.4 g) and placenta (0.4 g). (Scale bar: 100 μm.)

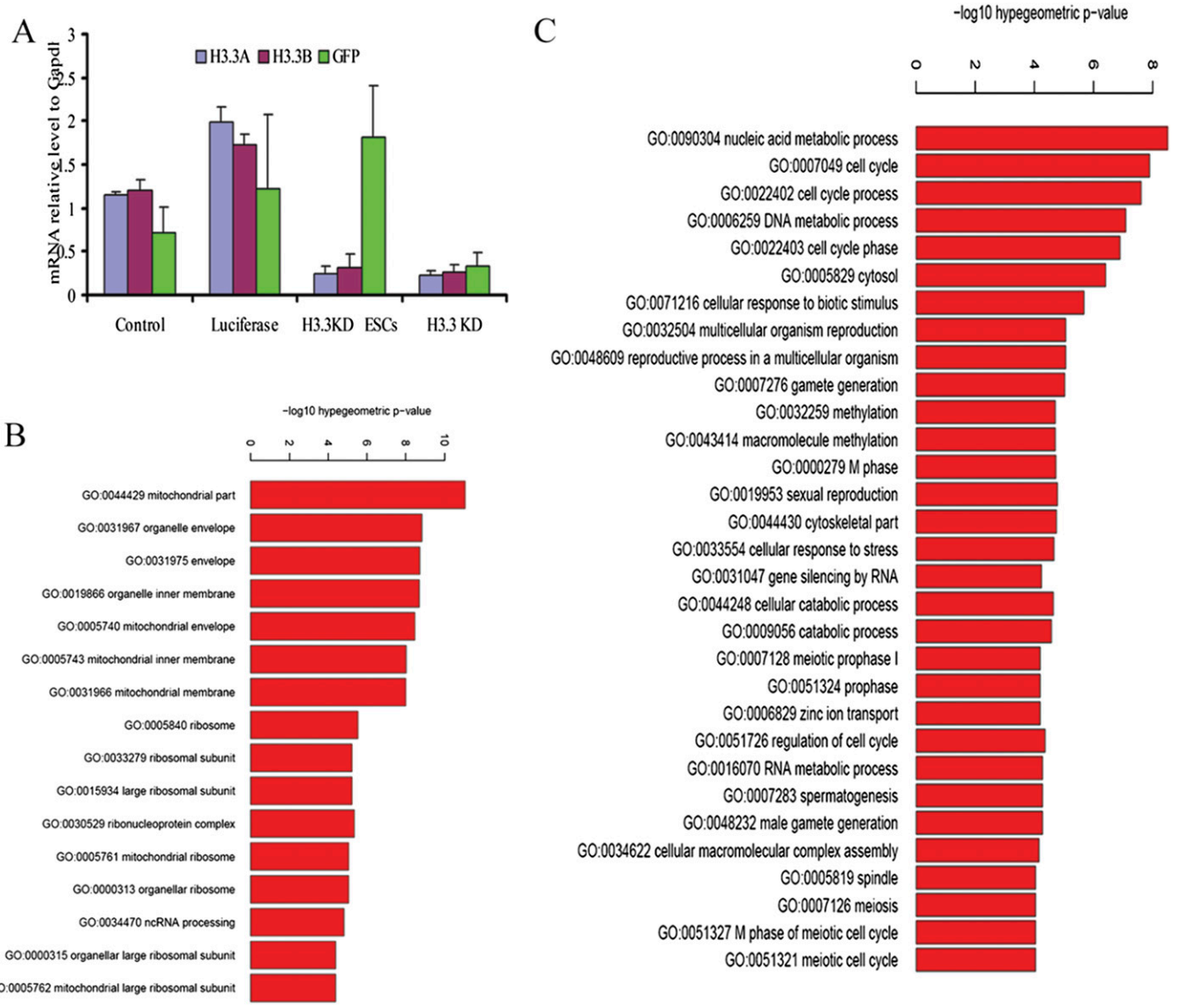

Fig. S4. (*A*) Quantitative RT-PCR results of the H3.3 and GFP transcription levels in Oct4-EGFP MEF SCNT morula embryos, showing that H3.3A and H3.3B are knocked down and the GFP levels also decreased in Oct4-EGFP MEFs SCNT embryos, but not in the ES cell nuclear transfer embryos. (*B*) Pathway analysis of the 1,387 genes that rescued in H3.3 addback was enriched with cell cycle, gamete generation, and methylation. (*C*) Pathway analysis of the up-regulated genes that were also down-regulated in H3.3-addback embryos revealed that they are mitochondrial and ribosome genes.

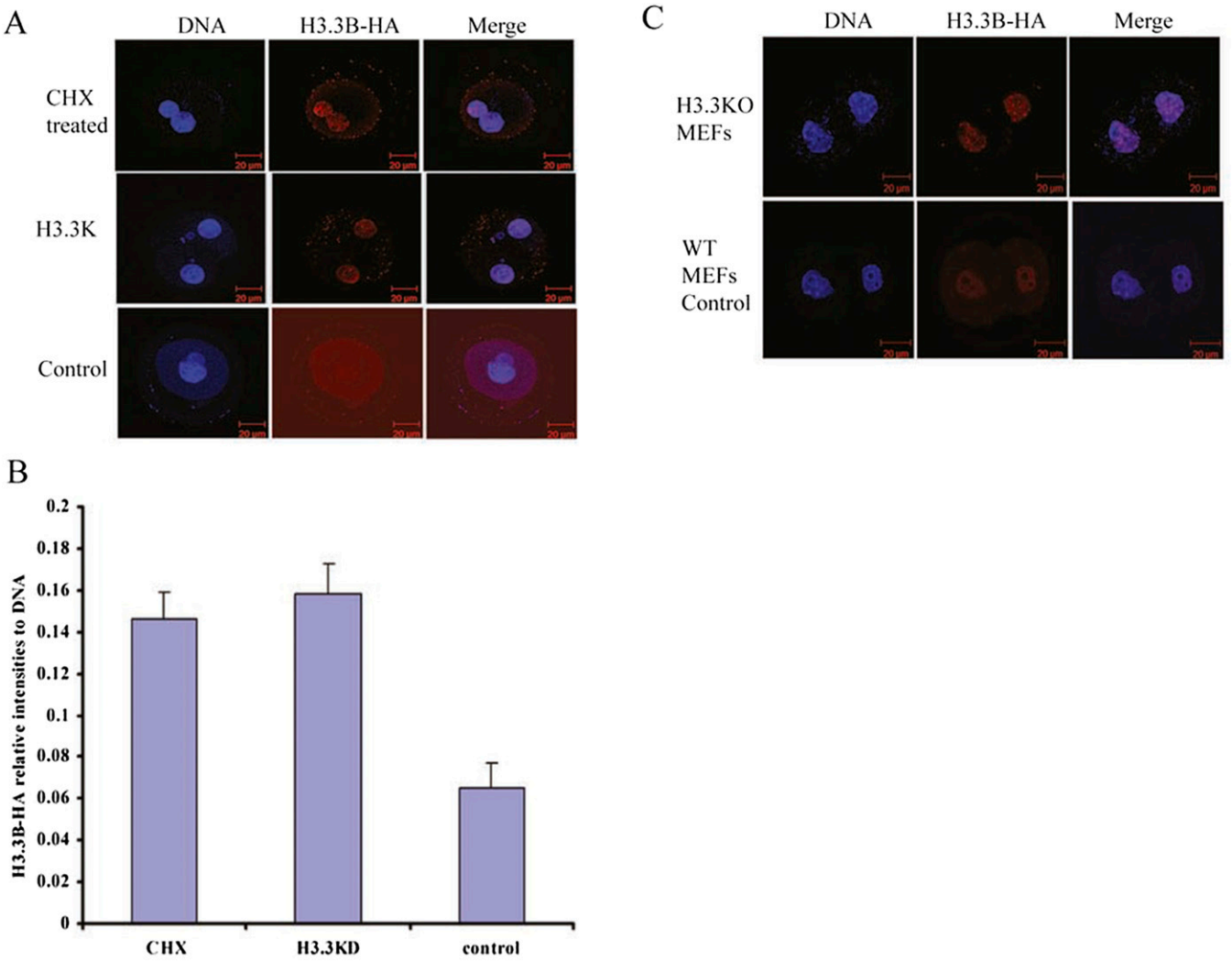

Fig. S5. (*A* and *B*) Embryos were produced by transfer of H3.3B-HA cumulus nuclei into WT enucleated oocytes. Cycloheximide (CHX) treated: the donor nucleus-derived H3.3B-HA is detectable by HA staining 20 h after activation, when the embryos were treated with the protein synthesis inhibitor cycloheximide for 5 h (10 μg/mL), whereas the donor-cell derived H3.3B-HA was almost undetectable in control SCNT embryos. H3.3KD: knockdown of maternal H3.3 blocked the loss of donor nucleus-derived H3.3B-HA in SCNT embryos. (*C*) SCNT embryos produced by transfer of H3.3-null nucleus (H3.3KO MEFs) into H3.3B-HA enucleated oocyte. The maternal H3.3B-HA is detectable in the nuclei of H3.3KO SCNT embryos 20 h after activation, indicating that the presence of H3.3 in the donor nuclei is not required for the deposition of maternal H3.3.

Table S1. Developmental potentials of H3.3KD parthenogenetically activated embryos

Embryo	No. oocytes	No. two-cell (%)	No. four-cell (%)	No. morula (%)	No. blastocysts (%)
Control	216	213 (98.6)	212 (98.1)	211 (97.7)	201 (93.1)
Luciferase	314	313 (99.7)	308 (98.1)	297 (94.6)	270 (86.0)
KD-A	228	226 (99.1)	219 (96.1)	211 (92.5)	184 (80.7)
KD-B	170	164 (96.5)	164 (96.5)	161 (94.7)	137 (80.6)
KD-AA	71	70 (98.6)	68 (95.8)	67 (94.4)	45 (63.4)*
KD-BA	366	349 (95.4)	320 (87.4)	274 (74.9)*	80 (21.9)**
KD+H3.3mRNA	119	116 (97.5)	116 (98.3)	110 (92.4)	80 (67.2)*
KD+H3.2mRNA	147	146 (99.3)	142 (99.6)	120 (81.6)	40 (27.2)**

Control, normal PA embryos; KD-A (knockdown H3.3A), PA embryos from oocytes only knockdown of H3.3A (4 μM each set, $n = 2$ sets); KD-B (knockdown H3.3B), PA embryos from oocytes only knockdown of H3.3B (4 μM each set, $n = 2$ sets); KD-AA (knockdown H3.3 after activation), PA embryos from oocytes that were injected with siRNAs 12 h after activation for knockdown of both H3.3A and H3.3B (4 μM each set, n = 4 sets); KD-BA (knockdown H3.3 before activation), PA embryos from oocytes that were injected with siRNAs before activation for knockdown of both H3.3A and H3.3B (4 μM each set, $n = 4$ sets); KD+H3.3mRNA, PA embryos from oocytes that were knocked down with H3.3 siRNA (4 μM each set, $n = 4$ sets) and addback exogenous H3.3mRNA (30 ng/μL); KD+H3.2mRNA, PA embryos from oocytes that were knocked down with H3.3 siRNA (4 μM each set, $n = 4$ sets) and addback exogenous H3.2-HA mRNA (30 ng/μL); luciferase, PA embryos from oocytes injected with siRNA target to luciferase (16 μM). *$P < 0.05$ and **$P < 0.01$, χ^2 test (statistic significance of differences was determined by comparing vs. luciferase).

Table S2. Developmental potentials of cumulus cell nuclear transfer embryos

Embryo	No. oocytes	No. two-cell (%)	No. four-cell (%)	No. morula (%)	No. blastocysts (%)
Control	598	565 (94.5)	378 (63.2)	302 (50.5)	184 (30.8)
H3.3 KD	405	311 (76.8)	169 (41.7)*	61 (15.1)*	9 (2.2)*
Luciferase	97	80 (82.5)	61 (62.9)	57 (58.8)	35 (36.1)
H3.3KD+H3.3mRNA	315	279 (88.6)	169 (53.7)*	129 (41.0)	79 (25.1)
H3.3KD+H3.2mRNA	156	115 (73.7)	54 (34.6)*	29 (18.6)*	7 (4.5)*
H3.3 mRNA	193	182 (94.3)	117 (60.6)	103 (53.4)	51 (26.4)

Control, cumulus nuclear transfer (SCNT) embryos; H3.3KD, cumulus SCNT embryos injected with siRNAs against both H3.3A and H3.3B in the oocytes (4μM each set, $n = 4$ sets, total concentration is 16 μM); H3.3KD+H3.3mRNA, cumulus SCNT embryos injected with exogenous H3.3 mRNA (30 ng/μL) into H3.3KD (4 μM each set, $n = 4$ sets) oocytes; H3.3KD+H3.2mRNA, cumulus SCNT embryos injected with exogenous H3.2 mRNA (30 ng/μL) into H3.3KD (4 μM each set, $n = 4$ sets) oocytes; H3.3 mRNA, cumulus SCNT embryos injected with exogenous H3.3 mRNA (30 ng/μL) ; luciferase, cumulus nuclear transfer embryos injected with siRNAs against luciferase (16 μM) served as H3.3 siRNA control. Percentages based on the number of oocytes. *$P < 0.01$, χ^2 test (statistical significance of differences was determined by comparing vs. luciferase).

Table S3. Derivation of ES cell lines from floxed h3f3a mouse blastocysts

No. blastocysts	No. ES cell lines derived (%)	No. WT ES cell lines (%)	No. heterozygous ES cell lines (%)	No. of homozygous ES cell lines (%)
49	39 (79.6)	10 (25.6)	22 (56.4)	7 (17.9)

Embryos were recovered from a superovulated h3f3a$^{flox/+}$ female mated with h3f3a$^{flox/+}$ male 40 h after hCG injection. Embryos were cultured in KSOM to blastocysts and plated individually in ES cell derivation medium in 96-well plate. ES cell derivation was described in the derivation of ES cell lines in the full method.

Table S4. Derivation of H3.3KO ES cell lines from blastocysts derived by nuclear transfer with H3.3KO embryonic fibroblast cells (i.e., MEFs)

Donor cell type	Oocyte manipulated	No. of two-cell embryos (%)	No. of morula (%)	No. of blastocysts (%)	No. ES cell lines derived (%)†
H3.3KO MEFs	141	80 (56.7)	29 (20.6)	13 (9.2)	4 (30.8)*
WT MEFs	113	68 (60.2)	36 (31.9)	24 (21.2)	6 (85.7)

Embryos produced by nuclear transfer of embryonic fibroblast cells (passage 1) isolated from E13.5 H3.3KO chimeric embryos. Blastocysts identified by YFP positive or negative from H3.3KO or WT MEFs SCNT embryos were cultured in medium composed of knockout serum replacement supplemented with PD98059 and LIF for ES cell derivation. ES cell lines were established by expansion of the ICM outgrowths from 96-well plate to six-well plate and characterized thereafter for stable lines. *$P < 0.01$, χ^2 test.
†No. of ES cell lines divided by number of blastocysts × 100.