SUPPLEMENTAL INFORMATION

SUPPLEMENTAL MATERIALS AND METHODS

Collection and culture of oocytes and embryos

Metaphase-II-stage eggs were obtained by superovulating 3-week-old C57BL6/J or B6D2F1 female mice (SLC Japan, Shizuoka) that had been treated with 5 IU of pregnant mare serum gonadotropin (PMSG; ASKA Pharmaceutical Co., Tokyo) followed 48 h later by 5 IU of human chorionic gonadotropin (hCG; ASKA Pharmaceutical). B6D2F1 mice were used for all experiments except for RNA sequencing that used C57BL6/J mice.

MII eggs were collected from ampullae of oviducts 15 h post-hCG injection and transferred to human tubal fluid medium (HTF) (1) supplemented with 10 mg/ml BSA (Sigma-Aldrich, Saint Louis, MO). MII eggs from B6D2F1 and C57BL6/J female mice were inseminated with spermatozoa obtained from the caudal epididymides of adult ICR and C57BL6/J male mice, respectively (SLC Japan) in HTF supplemented with 10 mg/ml BSA. Before insemination, spermatozoa were initially incubated for 2 h in TYH medium (2) or HTF medium supplemented with 10 mg/ml BSA for MII eggs of C57BL6/J female mice or B6D2F1 female mice, respectively, in an atmosphere of 5% CO_2/95% air at 38°C. Between four and six hours post-insemination, the fertilized eggs were washed and cultured in potassium simplex optimized medium (KSOM) (1).

Full-grown oocytes were collected from ovaries of 8-week old B6D2F1 female mice treated with 5 IU of PMSG 48 h earlier. The ovaries were transferred to HEPES-buffered KSOM containing 0.2 mM 3-isobutyl-1-methylxanthine (IBMX) to inhibit the spontaneous resumption of meiosis and punctured with a 27-gauge needle.

www.pnas.org/cgi/doi/10.1073/pnas.1804309115

Oocytes were transferred to IBMX-free α-minimal essential medium (α-MEM; Life Technologies, Inc.,Grand Island, NY) containing 5% fetal bovine serum and 10 ng/ml epidermal growth factor (both from Sigma–Aldrich) to permit resumption of meiosis and cultured for 16 h.

Treatment with DRB

To transiently inhibit minor ZGA, 1-cell embryos were cultured in KSOM containing 80 μM DRB (Sigma-Aldrich) between 4 and 20 hpi, and the treated embryos were then washed six times in KSOM without DRB and then cultured in KSOM without DRB. For transient inhibition of major ZGA, 2-cell embryos were cultured in KSOM containing 80 μM DRB between 26 and 42 hpi. Control embryos were cultured in DRB-free medium that contained the appropriate amount of dimethyl sulfoxide (DMSO), the solvent used to prepare the DRB stock solution.

***In vitro* transcription assay**

Transcriptional activity was measured by incorporation of BrU into nascent RNA as described previously (3). The plasma membrane of embryos was permeabilized with 0.05% of Triton X-100 (Sigma-Aldrich) in a physiological buffer (4) for 1 min at room temperature. The embryos were briefly washed 3 times with a physiological buffer and the incubated in a physiological buffer containing nucleotides and 0.4mM BrUTP (Sigma) for 15 min at 33 ºC. The transcription reaction was terminated by permeabilizing the nuclear membrane in a physiological buffer containing 0.2% of TritonX-100 for 3 min at room temperature. The permeabilized embryos, which were fixed in a physiological buffer containing 3.7% of paraformaldehyde (Wako, Osaka, Japan) for 1 h at room temperature, were subjected to immunocytochemistry using anti-BrdU antibody (1:50 dilution; Roche Diagonostic, Indianapolis, IN), which

has the cross reactivity against BrU, and Alexa flour 488-conjugated anti- mouse IgG as a secondary antibody (1:100 dilution; Sigma-Aldrich). The fluorescent signal was detected with a confocal laser-scanning microscope (LSM 5 EXCITER: Carl Zeiss MicroImaging GmbH, Oberkochen) and quantified by using a NIH Image program (Image J), as previously described (5).

Immunocytochemistry

Phosphorylated Ser2 of CTD: Embryos were fixed in PBS containing 2% paraformaldehyde for 15 min at room temperature. The fixed embryos were permeabilized with 0.5% Triton X-100 diluted in PBS for 15 min at room temperature. The embryos were then washed three times with PBS containing 1% BSA (Sigma), next incubated with anti-phosphorylated Ser2 of Pol II antibody (Abcam, Cambridge, UK, Cat#ab24758 or Cat#ab5095) in PBS containing 1% BSA (1:100 dilution) for 1 h at room temperature. The embryos were washed with PBS containing 1% BSA and incubated with FITC (fluorescein)-conjugated anti-mouse IgM secondary antibody (1:100 dilution; Abcam) or fluorescent-conjugated secondary antibody (1:100 dilution: fluorescein (FITC) AffiniPure Donkey Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch Inc., PA, Cat#711-095-152)for 1 h at room temperature.

SC35 and H3K4me3: Embryos were fixed with 4% paraformaldehyde for 15 min at room temperature. After being washed with PBS, embryos were permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature and then treated with an antibody against SC-35 (Sigma-Aldrich, Cat#S4045) or H3K4me3 (Millipore, Billerica, MA, Cat#07-473) overnight at 4ºC. The primary antibody was detected following a 1 h incubation at room temperature with a fluorescent-labeled secondary antibody (Alexa fluor 488 conjugated anti-mouse IgG (Life Technologies Corp., Carlsbad, CA,

Cat#A11001).

H3K9ac: Embryos were fixed in PBS containing 3.7% paraformaldehyde for 1 h at room temperature. The fixed embryos were permeabilized with 0.5% Triton X-100 diluted in PBS for 15 min at room temperature and then treated with an antibody against H3K9ac (1:100 dilution; Cell Signaling Technology, Danvers, MA, Cat##9671S) overnight at 4ºC. The primary antibody was detected following a 1 h incubation at room temperature with a fluorescent-labeled secondary antibody (1:100 dilution; Fluorescein (FITC) AffiniPure Donkey Anti-Rabbit IgG (H+L, Jackson ImmunoResearch Inc., PA, Cat#711-095-152).

H3K27me3: Embryos were fixed in PBS containing 3.7% paraformaldehyde and 0.2% Triton X-100 for 20 min at room temperature. They were treated with an antibody against H3K27me3 (1:500 dilution; Millipore, Cat##07-449) overnight at 4ºC. The primary antibody was detected following a 1 h incubation at room temperature with a fluorescent-labeled secondary antibody (1:100 dilution: Fluorescein (FITC) AffiniPure Donkey Anti-Rabbit IgG (H+L, Jackson ImmunoResearch Inc., Cat#711-095-152).

H3K36me3: Embryos were fixed in PBS containing 5% paraformaldehyde, 0.04% Triton X-100, 0.3% Tween 20 and 0.2% sucrose for 20 min at room temperature. The fixed embryos were permeabilized with 0.5% Triton X-100 containing PBS for 20 min at room temperature and then treated with an antibody against H3K36me3 (1:250 dilution: Abcam, Cat#9050) overnight at 4ºC. The primary antibody was detected following a 1 h incubation at room temperature with a fluorescent-labeled secondary antibody (1:100 dilution: Fluorescein (FITC) AffiniPure Donkey Anti-Rabbit IgG (H+L, Jackson ImmunoResearch Inc., Cat#711-095-152).

In each case, the cells mounted on a glass slide using VectaShield (Vector

Laboratories, Burlingame, CA, USA) supplemented with 3 mg/ml 4',6-diamidino-2-phenylindole (DAPI; Dojindo Laboratories, Kumamoto, Japan). Confocal digital images were collected using a confocal laser-scanning microscope (LSM 5 EXCITER: Carl Zeiss MicroImaging GmbH, Oberkochen).

Fluorescence recovery after photobleaching (FRAP)

FRAP analysis was conducted as described previously with slight modification (6). Briefly, after photobleaching, images were taken at 5 s intervals for 60 s. The mobile fraction (MF), an index of the rate of fluorescence recovery, was calculated using the following equation (38-40) : $MF = (F_{end}-F_{post})/(F_{pre}-F_{post})$, where F_{end} is the relative intensity of fluorescence at the endpoint, F_{post} is immediately after photo-bleaching, and F_{pre} is before photo-bleaching.

RNA extraction and preparation of the RNA-seq library

Two-cell C57BL/6J embryos transiently treated with DRB were collected 32 hpi and subjected to RNA extraction for RNA sequencing. Total RNA was extracted from 4,500 of embryos using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. RNA quality was verified on a Bioanalyzer RNA Pico Chip (Agilent Technologies, Santa Clara, CA). RNA-Seq library was generated as described previously (7) using the mRNA-Seq Sample Preparation Kit (Illumina, San Diego, CA) without selection of polyadenylated RNA. The RNA-seq library sequenced using a Genome Analyzer IIx (Illumina) and 35-nt single-end reads were mapped on the mouse genome reference sequence (mm9, UCSC Genome Browser) allowing for no mismatch using the ELAND software. The uniquely mapped reads on RefSeq genes were used to calculate RPKM values. The sequencing data have been deposited in the DDBJ Sequence Read Archive (DRA)

(http://trace.ddbj.nig.ac.jp/dra/index.html) under accession number DRA006557. RNA sequencing data of oocytes, 1-cell and 2-cell stage embryos treated without DRB were obtained from our previous work (7).

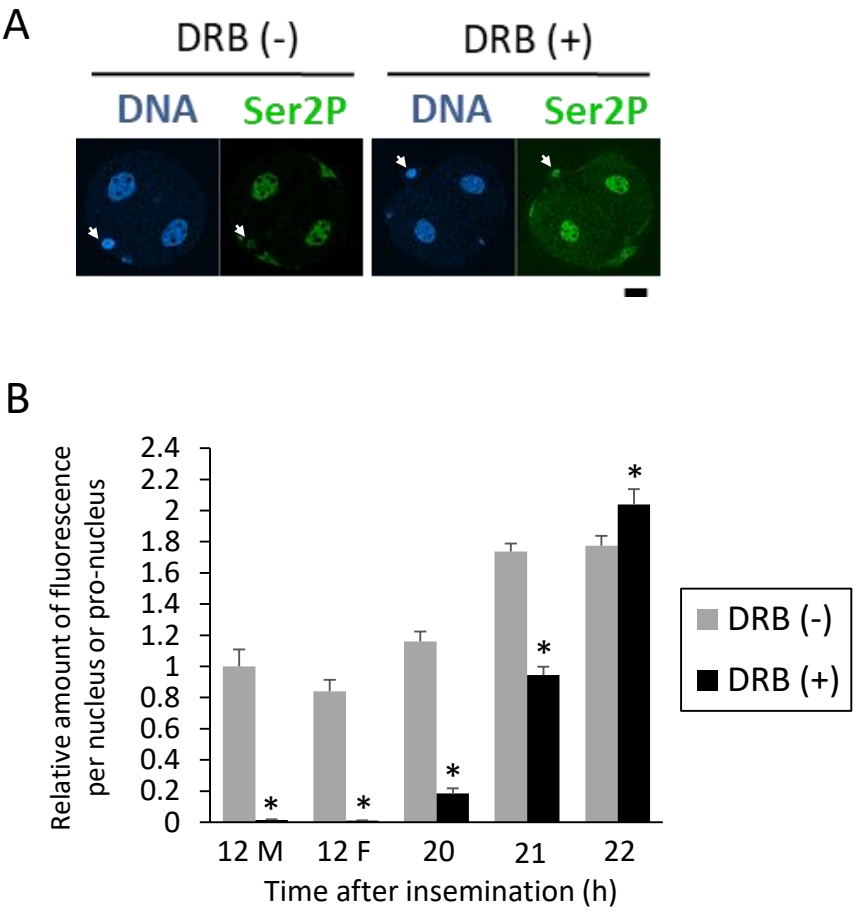
Reverse transcription and polymerase chain reaction

One hundred MII eggs or embryos were transferred to Isogen (Nippon Gene) including 50 pg of rabbit globin mRNA as an external control and total RNA isolated according to the manufacturer's instructions. Total RNA was treated with RQ1 RNase-Free DNase (Promega, Madison,WI) and prepared for reverse-transcription using a PrimeScript RT-PCR kit (Takara Bio Inc., Otsu), according to the manufacturer's instructions. PCR was performed in a thermal cycler (iCycler; Bio-Rad, Berkeley, CA) using Ex Taq DNA polymerase (Takara) and following primer pairs: Rabbit *α-globin*: 5'-GTGGGACAGGAGCTTGAAAT-3' and 5'-GCAGCCACGGTGGCGAGTAT-3', and *MuERV-L*: 5'-TTCTCAAGGCCCACCAATAGT-3' and 5'-GACACCTTTTTTAACTATGCGAGCT-3', and *Nid2*: 5'-CACCGAGGACAGTTTCCATT-3' and 5'-CCAGTTACCAGGTGCTGGAT-3', and *Mfsd7c*: 5'-GTCCTTGCTTGGTCTCTTGC-3' and 5'-CTTCCTCTCGTGACCCTCAG-3', and *Klf5*: 5'-ACGTACACCATGCCAAGTCA-3' and 5'-GTGGGAGAGTTGGCGAATTA-3'. The PCR conditions were as follows: Rabbit *α-globin*: 26 cycles of 95ºC for 30 s, 58ºC for 30 s, and 72ºC for 60 s. *Mfsd7c*: 38 cycles of 95ºC for 30 s, 59ºC for 30 s, and 72ºC for 60 s. *MuERV-L*, *Nid2* and *Klf5*: 38 cycles of 95ºC for 30 s, 60ºC for 30 s, and 72ºC for 60 s.

References

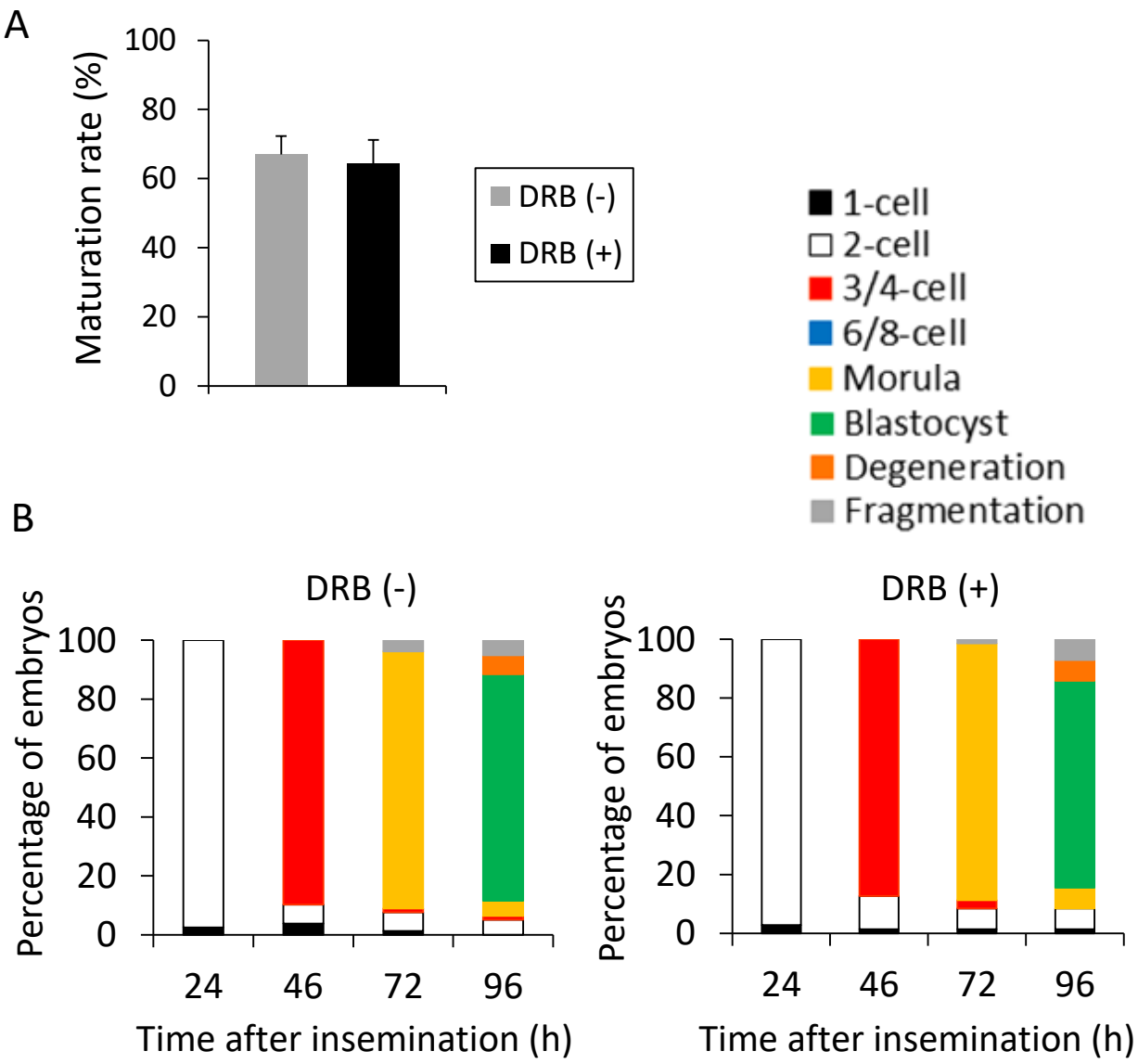
1. Lawitts JA, Biggers JD (1993) Culture of preimplantation embryos. *Meth. Enzymol.* 225, 153–64.
2. Toyoda Y, Yokoyama M, Hosi T (1971) Studies on the fertilization of mouse eggs invitro: I. in vitro fertilization of eggs by fresh epididymal sperm. *Jap. J. Anim. Reprod.* 16, 147-151.
3. Aoki F, Worrad D, Schultz R (1997) Regulation of Transcriptional Activity during the First and Second Cell Cycles in the Preimplantation Mouse Embryo. *Dev Biol* 181, 296–307.
4. Jackson DA, Hassan AB, Errington RJ, Cook PR (1993) Visualization of focal sites of transcription within human nuclei. *EMBO J.* 3, 1059-65.
5. Abe K, Inoue A, Suzuki MG, Aoki F (2010) Global gene silencing is caused by the dissociation of RNA polymerase II from DNA in mouse oocytes. *J Reprod Dev*. 56, 502-7.
6. Ooga M, Fulka H, Hashimoto S, Suzuki MG, Aoki F (2016) Analysis of chromatin structure in mouse preimplantation embryos by fluorescent recovery after photobleaching. *Epigenetics*, 11, 85-94.
7. Abe K, et al. (2015) The first murine zygotic transcription is promiscuous and uncoupled from splicing and 3' processing. *EMBO J.* 34, 1523–37.

Supplemental Fig. S1



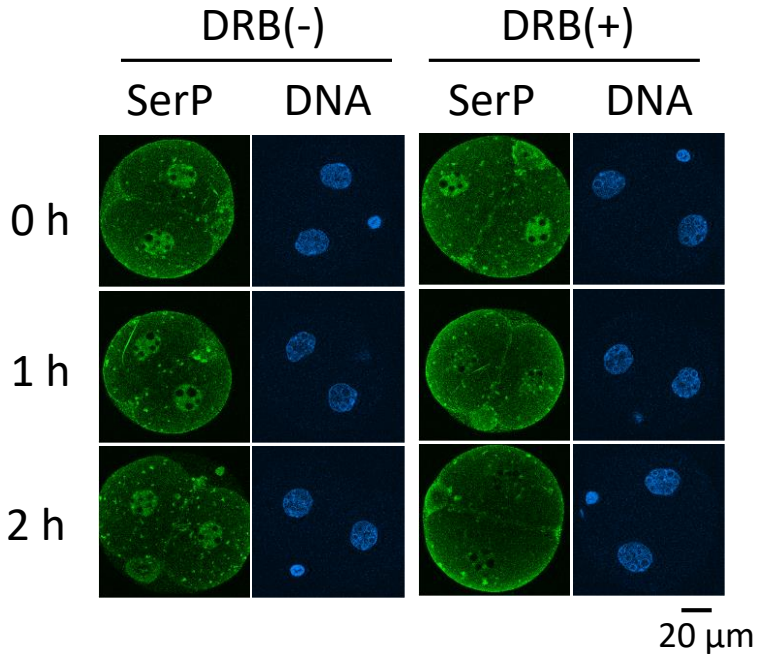
Supplemental Figure S1. Reversible effect of DRB on phosphorylation of RNA polymerase II. (A) Recovery of phosphorylation of RNA polymerase II on the C-terminal Ser2 (Ser2P). Embryos were cultured in DRB-containing medium between 4 and 20 hpi and then transferred to DRB-free medium. The embryos were collected 22 hpi. Scale bar corresponds to 20 µm. Arrowheads indicate polar body. **(B)** Quantification of Ser2P signal in the images of immunocytochemistry in (A) and Fig.1C. More than 20 pronuclei and nuclei were examined for each sample.

Supplemental Fig. S2



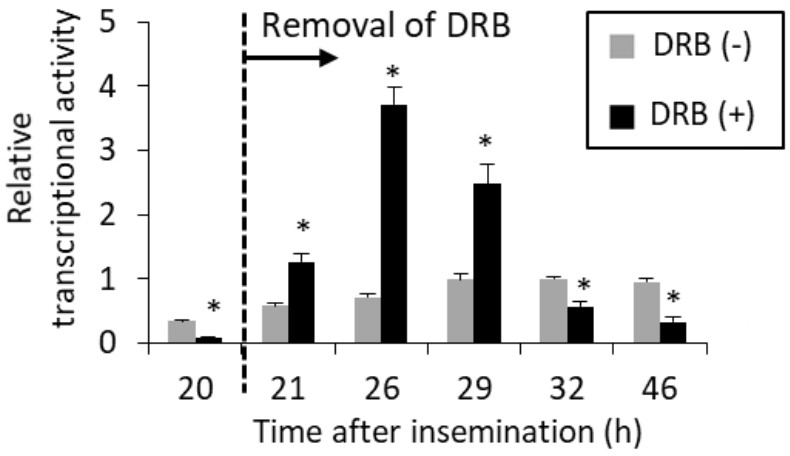
Supplemental Figure S2. Assessment of toxicity of DRB in oocytes. Full-grown oocytes were cultured in medium containing DRB during meiotic maturation for 16 h and then transferred to DRB-free medium. Oocytes that had reached MII were fertilized *in vitro* and observed for preimplantation development. **(A)** Percentage of matured oocytes extruding first polar bodies after incubation for 16 h. DRB (-) indicates oocytes cultured in medium containing dimethyl sulfoxide instead of DRB. The experiments were performed three times and >50 oocytes were analyzed for each experiment. **(B)** Development of embryos that had been cultured with DRB for 16 h before being fertilized *in vitro*. The y-axis shows percentages of embryos that developed to the indicated developmental stages. The experiment was performed three times and >17 embryos were analyzed for each experiment.

Supplemental Fig. S3



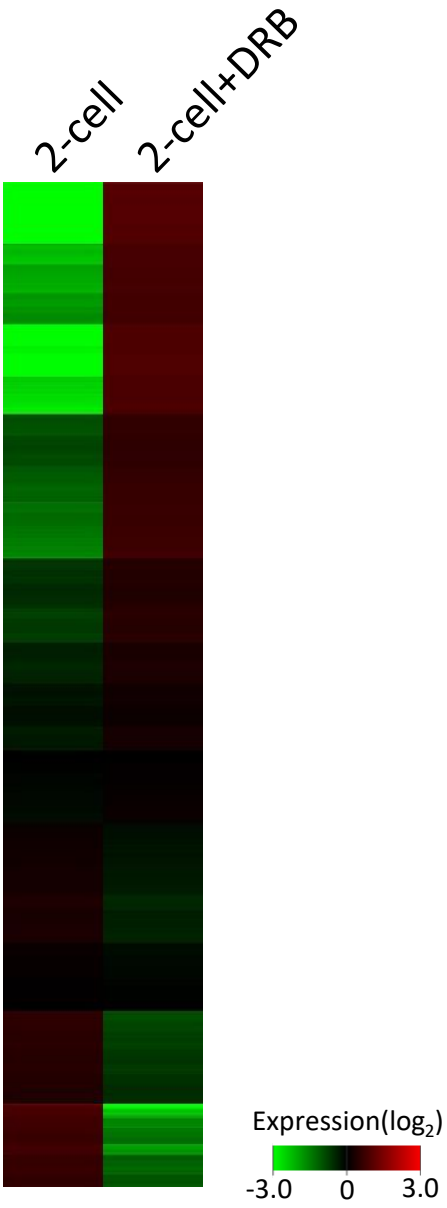
Supplemental Figure S3. **Inhibition of major ZGA by treatment of 2-cell embryos with DRB.** Two-cell embryos were transferred to medium containing DRB. One and 2 h after being transferred, the embryos were collected for detection of phosphorylated RNA polymerase II on the C-terminal Ser2 (Ser2P). Bar = 20 µm

Supplemental Fig. S4



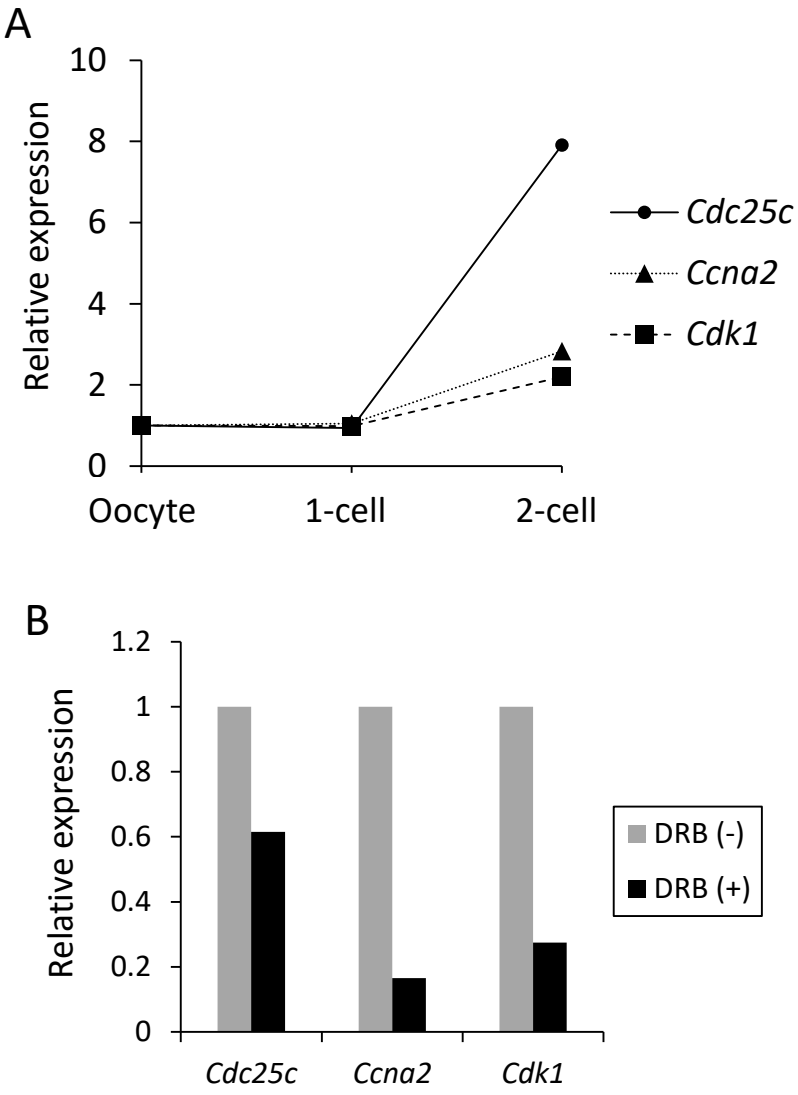
Supplemental Figure S4. Recovery of transcriptional activity after removal of DRB. Embryos were cultured with DRB between 4 and 20 hpi and then transferred to DRB-free medium and transcriptional activity measured at the indicated time points. Transcriptional activity in embryos not treated with DRB (DRB (-)) at 32 hpi was set as 1. The experiment was conducted three times and at least 20 nuclei were examined for each experiment. The data are expressed as mean ± SEM. Asterisks represent significant differences (by student's *t*-test; $P < 0.05$).

Supplemental Fig. S5



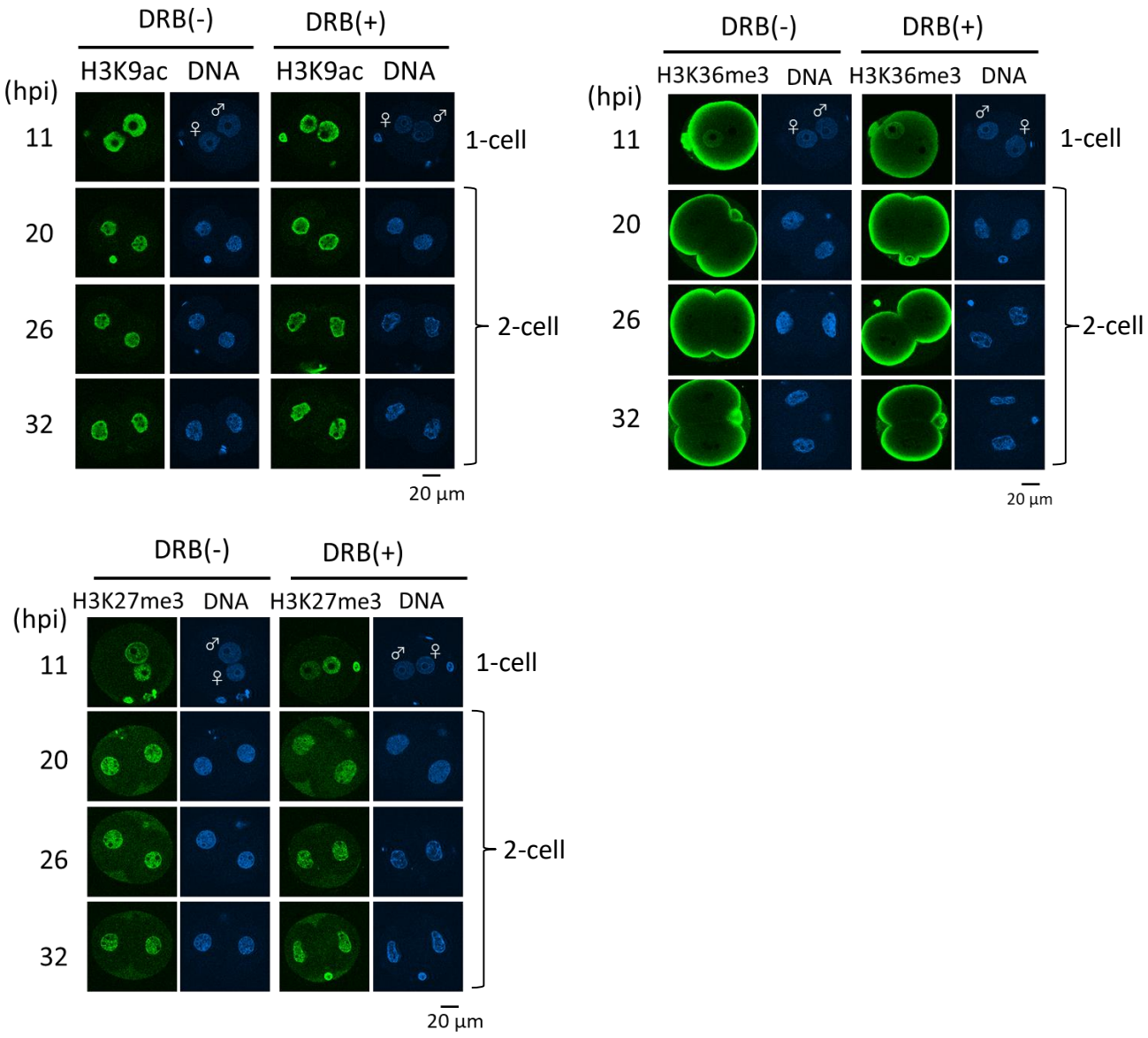
Supplemental Figure S5. Comparison of transcriptomes between 2-cell stage embryos treated with and without DRB. The heat map indicates differences of expression values of Refseq annotated genes in RNA-seq data between 2-cell stage embryos treated with and without DRB. Average values between 2-cell stage embryos treated with and without DRB were set as 1 and were converted into $\log_2$ values, and then data were used for hierarchical clustering.

Supplemental Fig. S6



Supplemental Figure S6. Repression of expression of mitosis-regulating genes by transiently inhibiting minor ZGA. (A) Relative expression levels of *Ccna2*, *Cdk1* and *Cdc25c* in MII eggs, and 1- and 2-cell embryos not treated with DRB. The RPKM (reads per kilobase per million) values were obtained from the RNA sequencing data and the values of MII stage oocytes were set as 1. **(B)** Relative expression levels of *Ccna2*, *Cdk1* and *Cdc25c* in the 2-cell embryos treated (DRB(+)) or not treated (DRB(-)) with DRB, respectively. The RPKM value of MII eggs was set as 1.

Supplemental Fig. S7



Supplemental Figure S7. Effect of DRB treatment on histone modifications. Embryos were cultured in DRB-containing medium between 4 and 20 hpi and then transferred to DRB-free medium. DRB (-) indicates embryos treated with dimethyl sulfoxide, a solvent of DRB, instead of DRB. The embryos were collected 32 hpi for immunocytochemical detection of H3K9ac, H3K27me3 and H3K36me3. The experiments were performed twice and similar results were obtained. Representative images are shown. Bar = 20 µm. Arrowheads indicate the polar body.