Supporting Information

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SI Experimental Procedures

AP Staining. AP staining was performed according to the manufacturer's instructions for the alkaline phosphatase kit (Beyotime). The ESC clones were fixed by cold acetone for 10 min and incubated with AP incubation medium containing 2% sodium glycerophosphate, 2% barbital sodium, 2% chloratum kalium, and 2% magnesium sulfate for 3 h at 37 °C and subsequently incubated in 5% cobaltous nitrate for 5 min and in 1% ammonium sulfide for 1 min. The results were observed under inverted microscope (DMi8, Leica).

Karyotype Analysis. Rat ESCs were arrested at metaphase by treatment with demecolcine (Sigma) for 4∼8 h. After incubation in 0.075 M KCl at 37 °C for 20 min, the cells were fixed in methanol-acetic acid (3:1), and then the cell suspension was dropped onto glass slides, stained with Giemsa, and used for G-binding analysis. More than 50 metaphase spreads were analyzed for each cell line. The photographs were captured under inverted microscope (DMi8, Leica).

The Genome Resequencing Data Analysis. The qualified amplifications of genomic DNA were used to construct Illumina libraries for high-throughput whole-genome sequencing. The whole-genome sequencing was performed by Illumina HiSeq 4000 sequencer with 150 bp paired-end sequencing reactions. The sequencing reads were aligned to the rat reference genome (rn6) by BWA software (28). After removal of duplicated reads, the read depth for each 500-kb bin was calculated and further normalized. The repeat regions were removed from the genome for the coverage calculation. The last scatterplot for the CNV was drawn by ggplot2.

Teratoma Formation. For teratoma formation, after being tripinsized, 2×10^6 rat ESCs were resuspended in 100 μL ESC medium and s.c. injected into SCID mice. Teratomas were collected after 6∼10 wk, and hematoxylin and eosin staining was performed following standard procedures. The photographs were obverted and captured under a bright-field light microscope.

RT-PCR. Total RNA was isolated using the RNeasy kit (QIAGEN) and cDNA (cDNA) made from 1,000 ng of RNA using SuperScript III (Invitrogen) and oligo-dT primers. For real-time PCR, we used SYBR premix Ex Taq (TAKARA). PCR mixtures were denatured at 94 °C for 5 min and cycled at 94 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s followed by final extension (Melt Curve detection) at 72–94 °C for 10 min after completion of 40 cycles. All of the gene expression levels were normalized by rat *Gapdh*. All of the primer sequences are listed in Table S3.

SSLP. Genomic DNA was isolated from the fetuses with Universal Genomic DNA Extraction Kit (TaKaRa). The SSLP analyses were performed for *D11mgh3*, *MYCR51*, *D12Mit2*, and *SCN2A* using PCR. PCR mixtures were denatured at 94 °C for 5 min and cycled at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s followed by a final extension at 72 °C for 10 min after completion of the 35 cycles. The PCR primer sequences for SSLP are shown in Table S3.

Immunofluorescence Microscopy Analysis. For immunofluorescence staining, rat ESCs were plated on glass coverslips coated by MEF cells and cultured for 3∼4 d. The ESC clones were fixed by 4% paraformaldehyde (Sigma) for 15 min and blocked with PBS + 0.1% BSA + 0.1% Triton (PBSBT) (Sigma) for 1 h and then incubated with primary antibodies overnight at 4 °C. Antibody localization was performed using a secondary antibody conjugated to fluorescein isothiocyanate and incubated at room temperature for 1 h in the dark. Specimens were observed with a fluorescent microscope. Primary antibodies included Oct4 (1:200; Santa Cruz), Sox2 (1:200; Millipore), Nanog (1:200; Millipore), and SSEA-1 (1:200; Millipore). Nuclei of ESCs were stained with Hoechest 33342 (Sigma). The photographs were captured under confocal microscopy (LSM 780; ZEISS).

Preparation of Donor ICM Cells. Rat ICM cells was isolated by immunosurgery as described with some modifications. The zona pellucida of rat blastocysts was removed using acidic Tyrode's solution (Sigma). The zona-free blastocysts were incubated with rabbit anti-rat IgG (Sigma), diluted 1:1 with DMEM (Invitrogen) medium added with 10% fetal bovine serum (FBS) (Invitrogen), for 30 min. Then, after being washed thoroughly, blastocysts were transferred to rat whole serum (Sigma), diluted 1:1 with DMEM/FBS medium, to complementation for 30 min. The outer dead TE cells were lysed and removed by gently pipetting the ICMs in a pipette. The ICMs were dissociated to single cells with 0.05% trypsin for 3∼4 min in 5% CO_2 at 37 °C.

Diploid Blastocyst Injection. The diploid blastocyst injection for chimera generation was performed as previously described (29). The blastocysts were recovered from the uterus of 4.5 dpc F344 female rats. Ten to fifteen ESCs were injected into the blastocysts, followed by transfer into the uterine horns of E3.5 pseudopregnant female SD rats. Chimeras and germ-line transmitted rats were identified by coat color. Germ-line transmission was observed by mating the chimeras with SD rats.

DNA Immuno-Dot Blot. DNA samples were diluted in TE buffer and denatured by heating at 100 °C for 10 min. Samples were rapidly chilled on ice for 5 min and then applied to a positive charged nylon membrane (PRN303B) under a vacuum using a 96-well Dot Blot Hybridization Manifold (Bio-Bot). The membrane was washed twice in 20× SSC buffer, dried for 30 min at 70 °C, and UV cross-linked. The membrane was blocked for 1 h with 5% BSA and probed with anti–50-methylcytosine antibody (dilution factor 1:250). The membrane was then washed twice with TBST before being probed with anti-mouse IgG secondary antibody conjugated to horseradish peroxidase. Following treatment with an enhanced chemiluminescence substrate, membranes were exposed with X-ray films (Carestream). Spot intensity was quantified using Image J image processing and analysis software (NIH).

RRBS Library Preparation and Data Analysis. Paired-end sequencing was performed on an Illumina HiSeq 2500 sequencer. Briefly, the sequencing reads were mapped to the rat genome (version rn6) by Bismark v0.13.1 (Babraham Bioinformatics) after trimming by Trim Galore (Babraham Bioinformatics) with the "-rrbs" option. The methylation levels of covered cytosine sites were calculated by dividing the number of reported C with the total number of reported C and T. CpG sites covered by more than 10 reads were used for the analysis. All of the heatmaps showed in the manuscript were plotted with the heatmap.2 function of R. Histograms of the methylation-level distribution were drawn by ggplot2. The discrete DNA methylation of CpG sites in ICRs was plotted and visualized by the Integrative Genomics Viewer (IGV) software (30).

Bisulfite Genomic Sequencing. Genomic DNA was extracted with MicroElute Genomic DNA Kit (TaKaRa). Bisulfite conversions and cleanup of bisulfite-converted DNA were done according

to the manufacturer's protocols (EpiTect Bisulfite Kit; Qiagen). *H19*-, *IG*-, *Gtl2*-, and *Snrpn*-DMRs (differentially methylated regions) were amplified with nested primers or seminested primers (Table S3). The first round of PCR was performed using 95 °C for 5 min and then 25 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 60 s. One microliter of the first-round PCR product was used as a template for the second-round PCR, which was performed with 95 °C for 5 min and then 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The PCR products were cloned into pMD18-T vectors (Takara). At least 10 randomly selected clones were sequenced and analyzed for unmethylated C-to-T conversion at each locus.

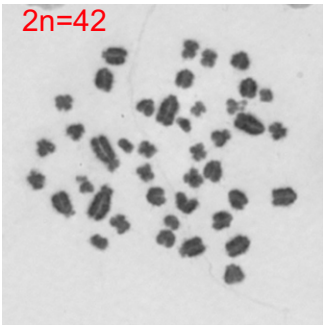

Fig. S1. Karyotype analysis of rat ESCs (related to Fig. 1). Shown is the karyotype analysis of rat ESC line DAESC-1 with a normal diploid 42-chromosome set.

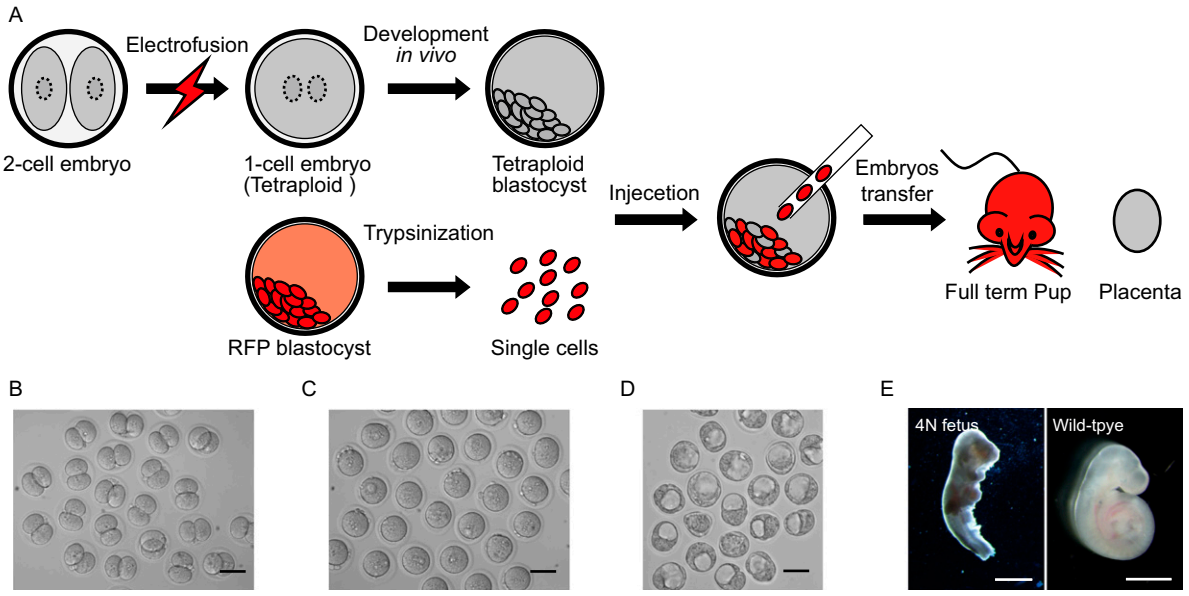

Fig. S2. Establishment of rat tetraploid complementation assay platform (related to Fig. 2). (*A*) Schematic overview of the procedure of producing pups by tetraploid complementation with ICM cells isolated from early blastocysts. (*B*) Morphology of two-cell stage embryos harvested from the oviduct of 1.5 dpc female SD strain rats. (Scale bar, 100 μm.) (*C*) Morphology of one-cell stage tetraploid embryos derived by electrofusion of two-cell stage SD embryos. (Scale bar, 100 μm.) (*D*) Tetraploid blastocysts obtained from in vivo transplanted electrofused two-cell stage rat embryos. (Scale bar, 100 μm.) (*E*) Morphology of a retarded E10.5 fetus produced by tetraploid complementation using DAESC-1 at passage 10. A wild-type E10.5 development fetus served as the control. (Scale bar, 500 μm.)

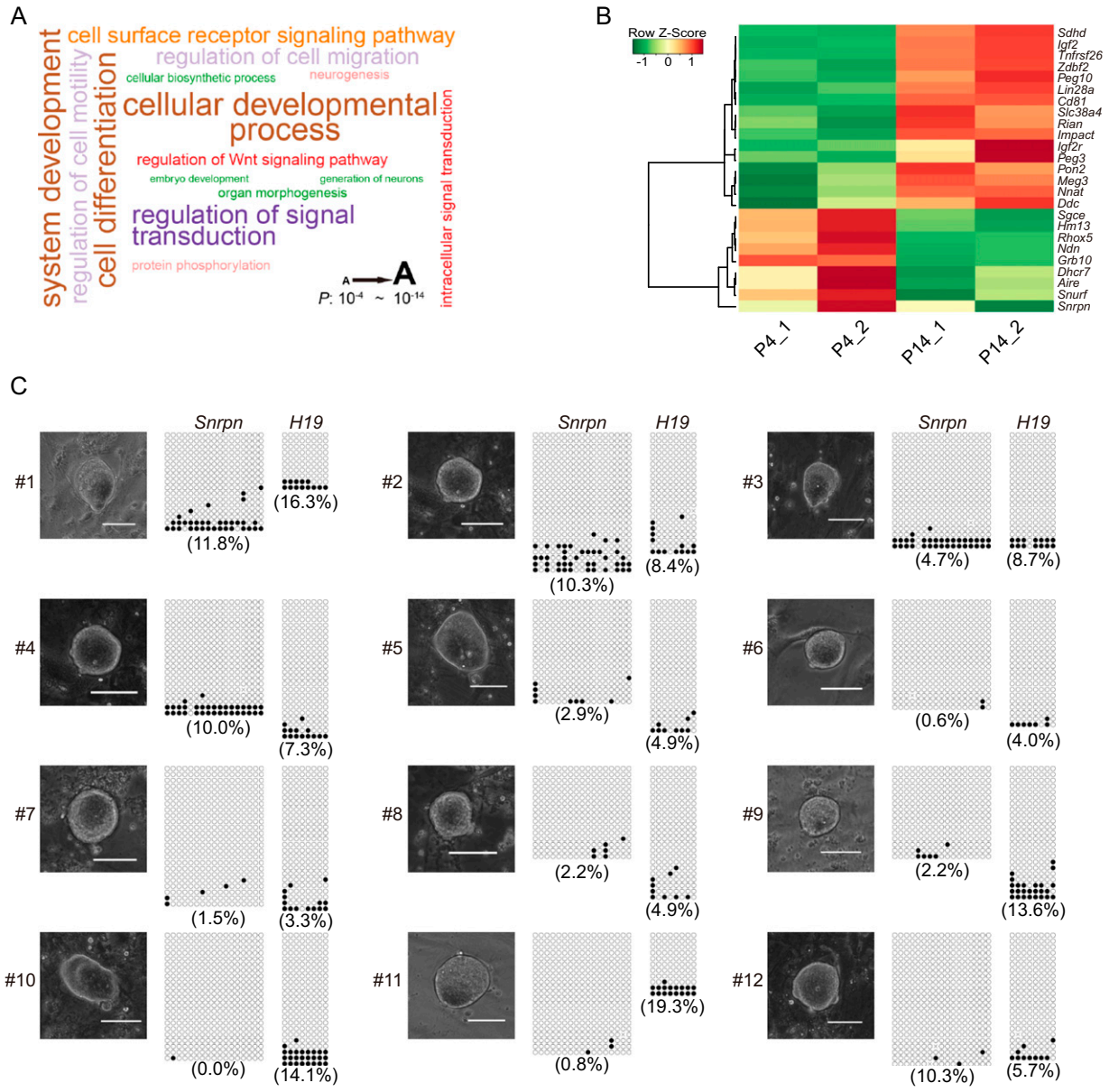

Fig. S3. Epigenetic instability of rat ESCs in different passages (related to Fig. 3). (*A*) The enriched Gene Ontology (GO) terms of differentially expressed genes between rat ESCs at passage 4 and passage 17. (*B*) Heatmap showing the expression levels of imprinted genes in rat ESCs at different passages (P4, passage 4; P14, passage 14). Twenty-five imprinted genes in total exhibited a differentially expressed tendency in rat ESCs between different passages. (*C*) Representative phase contrast image of subclones derived from rat ESC line DAESC-6 at passage 15 (*Left*) and their bisulfite sequencing of ICRs of *Snrpn* and *H19*, respectively (*Middle* and *Right*).

Table S1. Developmental potentiality of rat ESCs tested by diploid blastocyst injection and tetraploid complementation

			2N injection				4N injection		
								No. embryos arrested (%)	
Cell line	Background	Passage	Injected blastocysts	Live pups	Chimeras	Germ line	Injected	E7.5–E9.5	E9.5–E11.5
DAESC-1	DA	P10–P16	45	17	9	Yes	145	7 (4.8)	6 (4.1)
DAESC-2		P14-P16	70	11	4	Yes	110	8 (7.3)	3 (2.7)
DAESC-3		P13-P17	132	21	8	Yes	—	—	—
BSESC-1	BN × SD	P15, P16, P28	82	15	10	NA	—	—	—

BN × SD, Brown Norway strain rat mated with Sprague–Dawley strain rat; DA, Dark Agouti strain rat; NA, not available; NT, not tested.

Table S2. In vivo development of tetraploid blastocysts microinjected with ICM cells isolated from rat blastocyst

			Development in vivo (%)		
Rat strain	Injected	Recipient	Full-term	Live adult	Sex
BN × SD-RFP	24	2	3 (12.5)	3 (12.5)	Male/female
DA × DA	18	1	2 (11.1)	1 (5.6)	Male

BN × SD, Brown Norway strain rat mated with Sprague–Dawley strain rat; DA, Dark Agouti strain rat

Table S3. Primers used in the study (related to *Experimental Procedures*)

Assay	Primer name	5′-primer sequence-3′
RT-PCR	*Oct4*	GGGATGGCATACTGTGGAC
		CTTCCTCCACCCACTTCTC
	Nanog	GCCCTGAGAAGAAAGAAGAG
		CGTACTGCCCCATACTGGAA
	Sox2	GGCGGCAACCAGAAGAACAG
		GTTGCTCCAGCCGTTCATGTG
	Esrrb	GGCGTTCTTCAAGAGAACCA
		CCCACTTTGAGGCATTTCAT
	Gapdh	AAGGCTGTGGGCAAGGTCATC
		GTCATCATACTTGGCAGGTTTCTCC
SSLP	*D11mgh3-F*	GGATACAAATTGGAGCTGAAATACGA
	D11mgh3-R	AATACAAGTCATAGTGTCCTGCTGGC
	MYCR51sate-F	GACCAGCGTCACTGATAGTAGGGAGT
	MYCR51sate-R	CGTACCCCAATCCTGAACCACTC
	D12Mit2-F	GGATCTTGCCAAGGTGATGCTTG
	D12Mit2-R	CACTCGGCTTCTGAATGTATTGGAA
	SCN2Asate-F	CTGTTTCAGGTATGATTCGGGAACC
	SCN2Asate-R	TAATTGGCGATATCATTTCACTAACTGG
Bisulfite sequencing of DMRs	*rH19F*	AAGAGTATGGAGGATTATGTTTT
	rH19Ro	AAACCCATAATTATAAAATCATAA
	rH19Ri	TCAATTGCAATCTATTTCAACAAA
	rGtl2-F BS	AGAAATTTATTTATGGTGGGAGATATG
	rGtl2-Ri BS	TACCTATACCCCCTATTCCTTAACCC
	rGtl2-Ro BS	CCCTTTTCTACATTTCTCTTTTCCTTC
	ratIGDMR F0	GGTTTTTTGTGTTAGGGTAGTG
	ratIGDMR F1	TTTTGTGTATTTGTGTTATGGG
	ratIGDMR R1	AAAAACCACAATCCTTATACACA
	ratIGDMR R2	CCACAACAACTATACACAATCC
	rSnrpn Fo	GTAGTTAGAGAGTGATATGGATTTTTG
	rSnrpn Ro	CCGACATCCTAAAACCCAAAAACCATTC
	rSnrpn Fi	AGAGAGTAGAGGGTGTTGGAGATG
	rSnrpn Ri	ACCGTTCCAAAATACCTAAATCTAC