Supporting Information

Bogliotti et al. 10.1073/pnas.1716161115

SI Materials and Methods

Embryo Production and Processing. IVM-IVF embryos used in this study were produced as previously described (1). Briefly, bovine ovaries were collected from a local abattoir, and cumulus–oocyte complexes (COCs) were aspirated from selected follicles. COCs with intact cumulus cells were washed and placed in maturation medium for 24 h, after which IVF was performed. Fertilized embryos were denuded, and presumptive zygotes were cultured in KSOM (ZEBV-100; Zenith Biotech) supplemented with 4 mg/mL of BSA under mineral oil at 38.5 °C and 5% CO_2 in air until the blastocyst stage (day 7 after fertilization). Blastocysts were collected, and those that were not hatched were treated with 5 mg/mL of Pronase (10165921001; Sigma) for 3–4 min to remove the ZP (2). ZP-depleted blastocysts were washed five times in SOF·Hepes and were processed for ESC derivation.

OPU-IVF– and SCNT-derived embryos were produced by Trans Ova Genetics using their standard procedures and were shipped overnight to the University of California, Davis for bESC derivation.

IF. CTFR-bESCs (P11, P21, P24, P33, and P43) were allowed to grow to 60–70% confluence in a 12-well dish and were fixed using freshly prepared 4% paraformaldehyde in PBS for 10 min at room temperature. Then, cells were washed three times with PBS and blocked with PBST (0.3% Triton X-100 in PBS) supplemented with 3% (vol/vol) of normal donkey serum (NDS) (D9663; Sigma) for 30 min at room temperature. Cells were incubated with primary antibodies in PBST + 1% NDS for 1 h at room temperature and were washed three times for 10 min with gentle rotation using PBST + 1% NDS. After that, cells were incubated with fluorescent-labeled secondary antibodies at 1:500 dilutions for 1 h at room temperature. Cells were washed three times with PBST for 10 min each, and nuclei were stained using 0.01 μg/μL Hoechst 33342 solution (62249; Gibco) for 10 min. Cells were observed and visualized using a Nikon TE2000-U inverted microscope. The primary antibodies used in this study were anti-GATA6 (sc-7244; 1:300; Santa Cruz Biotechnology), anti-SOX2 (ANS79-5M; 1:300; BioGenex), anti-CDX2 (MU392A-UC; 1:300; BioGenex), and anti-POU5F1 (sc-8628; 1:300; Santa Cruz Biotechnology).

Karyotyping. CTFR-bESCs P34 were grown in a six-well plate to 50–60% confluence. The medium was changed, and 37.5 μL Demecolcine (200 ng/mL) (D7385; Sigma) per milliliter of medium was added to the wells and incubated at 37 °C for 1 h. Cells were harvested using TrypLE (12563011; Gibco) and were centrifuged at 300 × *g* for 5 min. The medium was aspirated, and 15 mL of hypotonic KCL solution (0.075 M in H_2O, prewarmed at 37 °C) was added drop by drop. Cells were incubated at 37 °C for 10 min, and then 10 drops of Carnoy's fixative (3:1 methanol: acetic acid) were added. Cells were centrifuged at 300 × *g* for 5 min, and the supernatant was discarded. Five milliliters of Carnoy's fixative was added to the cell pellet and incubated for 15 min. The fixation was repeated three times, and cells were resuspended in 100–300 μL of Carnoy's fixative after the last washing.

Slides were prepared by soaking them in chilled 70% ethanol and were rinsed with ice-chilled double-distilled H_2O. Slides were maintained at −20 °C until use. Cells were dropped at arm's length onto the clean, wet, chilled slides using a glass pipette. Slides were allowed to air dry for about 10–15 min before DNA staining. Giemsa staining was performed by soaking the slides in a solution of Giemsa stain (GS500; Sigma) diluted 1:20 in deionized water for 10 min. Slides were rinsed in deionized water and air dried. Stained slides were visualized using a Nikon TE2000-U inverted microscope at 1,000× magnification under oil immersion.

RNA-Seq. A confluent well of six-well plates of two CTFR-bESC lines (A: P13, P23, P35, and P45; B: P12, P24, P35, and P46) were used for RNA-seq. Whole blastocysts and fibroblasts were used as controls. Total RNA was isolated using the Qiagen RNeasy Mini Kit and then was reverse-transcribed using iScript RT Supermix (Bio-Rad). Libraries were constructed using the TruSeq RNA Sample Prep Kit (Illumina) and were sequenced on an Illumina HiSeq 2500 platform according to the manufacturer's instructions. Sequenced reads were mapped to the bovine UMD3.1 genome assembly and Ensembl 78 genebuild annotation using CLC Genomics Workbench 7.0 (CLC bio). RPKM values were calculated for each gene.

Teratoma Formation Assay. Briefly, immunodeficient NOD.*Cg-Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NOD *scid* gamma) mice were injected i.m. with about 1 million P10 CTFR-bESCs. Two different CTFR-bESC lines were tested. Teratomas were allowed to grow for 8 wk, and then mice were killed for tissue collection. Teratomas were fixed in 4% neutral-buffered formaldehyde, dehydrated through a graded series of alcohol/xylene, embedded in paraffin, sectioned, and subjected to H&E staining and IF analysis with lineage markers.

Preparation of CTFR-bESCs for ChIP-Seq. Two lines of CTFR-bESCs at P12 were separated from the MEFs using Feeder Removal MicroBeads (130-095-531; Miltenyi Biotech) following the manufacturer's protocol. Briefly, one well of a six-well plate was harvested using TrypLE (12563011; Gibco). First, MEFs were magnetically labeled, and then the cell suspension containing CTFR-bESCs and labeled MEFs was loaded onto a MACS LS Column placed in a magnet (MACS Separator). The magnetically labeled MEFs were retained within the column, while the unlabeled CTFR-bESCs were collected in the run through. CTFR-bESCs were counted and separated into tubes containing 20,000 cells. Cells were cross-linked in a final volume of 1 mL of PBS using 0.25% formaldehyde (28906; Pierce) in a rotating wheel (at 40 rpm) for 8 min at room temperature. Cross-linking was stopped by adding 125 mM of glycine (provided in the True MicroChIP Kit, C01010130), and tubes were placed in a rotating wheel at 40 rpm for 5 min at room temperature. Cells then were centrifuged for 10 min at 300 × *g* and 4 °C, and the supernatant was carefully removed. Cell pellets were washed with 1 mL of ice-cold PBS supplemented with phosphatase inhibitor cocktail (PIC) (provided in the True MicroChIP Kit, C01010130) and were centrifuged again for 10 min at 300 × *g* and 4 °C. The supernatant was removed, leaving about 10–20 μL of solution with the cells. Cells were directly processed for immunoprecipitation by snap-freezing in liquid nitrogen and were stored at −80 °C until use.

ChIP, Library Preparation, and Sequencing. Chromatin from CTFR-bESCs was prepared following the protocol recommended by the True MicroChIP manual (C01010130; Diagenode) with minimal modifications. Cross-linked cell pellets containing 20,000 P12 CTFR-bESCs (devoid of MEFs as described above) were lysed by adding 50 μL of complete lysis buffer (provided by the True MicroChIP kit) and incubation on ice for 5 min. After that, 70 μL of ice-cold HBSS supplemented with PIC (1×) was added to the cell lysates. The entire volume (~130 μL) was transferred to a

sonication AFA microTUBE presplit snap-cap vial (520045; Covaris) and was sonicated using a Covaris S220 ultrasonicator (500217; Covaris) with the following settings: duty cycle 5%, intensity 3, cycle/burst 200, number of cycles: 12 (60 s). Sonicated samples were transferred to a 1.5-mL low-adhesion tube, and 70 μL of ice-cold HBSS PIC was added per sample. Samples were centrifuged at 14,000 × *g* for 10 min at 4 °C, and the supernatant containing the sonicated chromatin was transferred to a new tube.

Sheared chromatin from 20,000 CTFR-bESCs was divided as follows: 10 μL for input, 100 μL for H3K4me3 immunoprecipitation, and 100 μL for H3K27me3 immunoprecipitation, making a final concentration of ∼10,000 cells per immunoprecipitation. Immunoprecipitation was done using the True MicroChIP Kit (C01010130; Diagenode) according to the manufacturer's instructions using 0.25 μg of anti-H3K27me3 antibody (ABE 44; Millipore) and 0.25 μg of anti-H3K4me3 antibody (provided with the kit). After de–cross-linking, DNA was purified using MicroChip DiaPure columns (C03040001; Diagenode) and was eluted in 11 μL of DNA elution buffer.

Sequencing libraries were prepared following the manufacturer's instructions using the ThruPLEX DNA-seq kit (R400406; Rubicon) with 16 cycles in the library-amplification step. Libraries were purified using XP AMpure beads (A63882; Agencourt). A 1:1 ratio of AMpure beads to sample volume was used and incubated for 10 min at room temperature. Washes were done with freshly prepared 80% ethanol and, after the last washing beads were allowed to air dry for about 5 min before eluting in 30 μL of molecular-grade water (W4502; Sigma). Libraries were quantified using the Qubit dsDNA HS Assay Kit (Q32851; Thermo Fisher Scientific) and were diluted to be run in a Bioanalyzer using a high-sensitivity DNA chip (5067-4626; Agilent Technologies). Libraries were sequenced at the Vincent J. Coates Genomics Sequencing Laboratory at the University of California, Berkeley in an Illumina HiSeq 4000 platform where sequencing was performed as 100-bp paired-end.

Raw reads were checked for sequencing quality using FastQC and then were aligned to the annotated bovine genome (UMD 3.1 assembly) using bwa-aln (3, 4). Peak calling was done using MACS2 (5) with narrow settings for H3K4me3 (-g 2.67e9 -q 0.01 -m 2 100 -B–SPMR) and broad settings for H3K27me3 (-g 2.67e9 -q 0.05 -m 2 100–broad -B–SPMR–fix-bimodal–extsize 200). Peaks were visualized using the Golden Helix GenomeBrowse tool (Golden Helix, Inc.; available at www.goldenhelix.com). Called peaks were further analyzed using HOMER (6) to find peak associations with gene features, and GO analysis was performed using DAVID (7, 8).

Population-Doubling Time. Two vials of cells stored at −80 °C containing CTFR-bESCs lines A and B at P13, P23, P35, and P45 were thawed in a warm water bath (37 °C) until only a small ice crystal was present. Cells were transferred to a 15-mL Eppendorf tube (∼1 mL), and 3 mL of culture medium was added to dilute the cryoprotectant. Cells were centrifuged for 5 min at 200 × *g*, and pellets were resuspended in CTFR medium and seeded in wells containing MEFs in the presence of 10 μM of the ROCK inhibitor Y27632 for 24 h. CTFR-bESCs were grown at 37 °C and 5% CO_2 for 1 wk, and medium was changed daily. After 1 wk, cells were trypsinized and were counted using an automated cell counter (TC20; Bio-Rad), and about 70,000 live cells per passage (5% of a well in a 12-well plate) were plated in two 24-well plates in the presence of the ROCK inhibitor Y27632.

Four time-points (days 2, 3, 4, and 5) per passage were evaluated by cell counting using the automated cell counter mentioned above. After the cell numbers were fitted to a growth curve, the population-doubling time was calculated along the logarithmic growth phase of the curve (day 2 to day 4).

AP Staining. CTFR-bESCs (P13) were grown in a four-well plate to 60–70% confluence. Detection of AP activity was performed using the Alkaline Phosphatase Staining Kit II (00-0055; Stemgent) following the manufacturer's protocol. Briefly, CTFR-bESCs were washed in PBS supplemented with 0.05% Tween-20 and fixed for 4 min at room temperature using Fix Solution (provided with the kit). Fixed cells were washed once in PBS supplemented with 0.05% Tween-20 and incubated with freshly prepared AP Substrate Solution (provided with the kit) in the dark at room temperature for 15 min. To stop the reaction, AP Substrate Solution was removed, and cells were washed twice with PBS. After staining, cells were observed in a Nikon TE2000-U inverted microscope, and images were captured using RI Viewer Imaging Software at 10×.

Cell-Cycle Analysis by Flow Cytometry. CTFR-bESCs (P24) were grown in a 12-well plate to 70–80% confluence and were dissociated using TrypLE Express Enzyme (12563011; Gibco). After dissociation, 1×10^6 cells were aliquoted in a 1.5-mL Eppendorf tube, washed twice with PBS supplemented with 2% FBS and 0.1% BSA, and centrifuged at 200 × *g* for 5 min. The supernatant was discarded carefully without disturbing the cell pellet, and 1 mL of ice-cold 70% ethanol was added dropwise with gentle vortexing to resuspend the pellet and minimize cell clumping. Fixed cells were stored at −20 °C until flow cytometry analysis. Propidium iodide (PI) staining was performed. Briefly, cells were centrifuged at 200 × *g* for 10 min and were washed first in PBS supplemented with 2% FBS and 0.1% BSA and second in Stain Buffer (554656; BD Pharmingen). After the second washing, 1×10^6 cells were resuspended in 500 μL of PI/RNase Staining Buffer (550825; BD Pharmingen) and were filtered through a 5-mL round-bottomed polystyrene tube with a cell-strainer snap cap (352235; Falcon). Cells were incubated in PI/RNase Staining Buffer for 15 min at room temperature, protected from light, and were analyzed within 1 h on a FACScan flow cytometer (Becton Dickinson) equipped with a 488-nm excitation laser. Cells were diluted to 1×10^6/mL in PBS to achieve a flow rate of ∼400 events/s. Data were acquired and analyzed using CellQuest Pro Software (Becton Dickinson). Cell clumps were gated out on a FL2-A/FL2-W dot plot, and 10,000 single events were collected per sample. Bovine fibroblasts were analyzed as controls.

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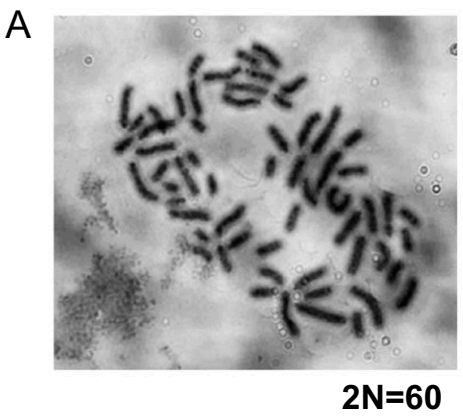
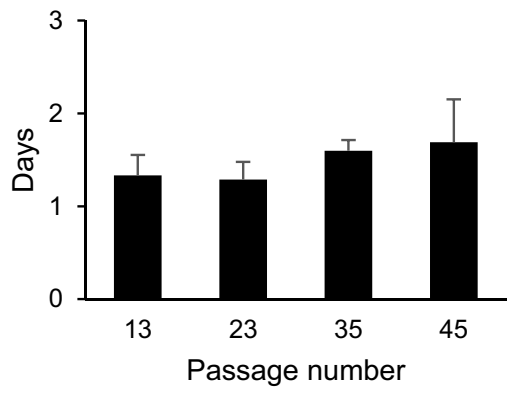
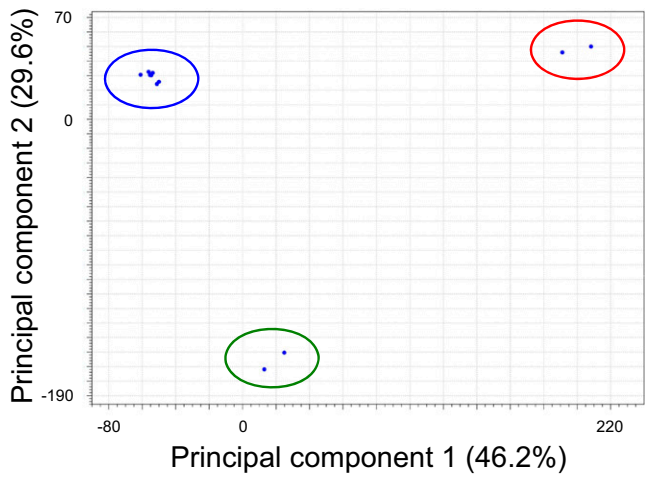
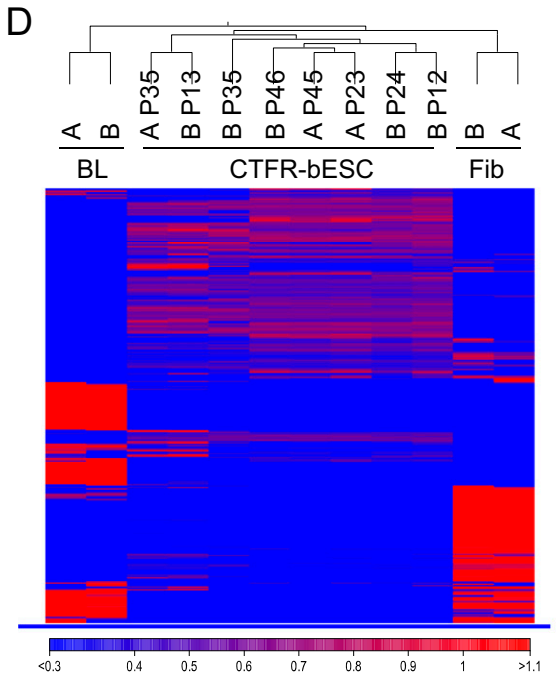


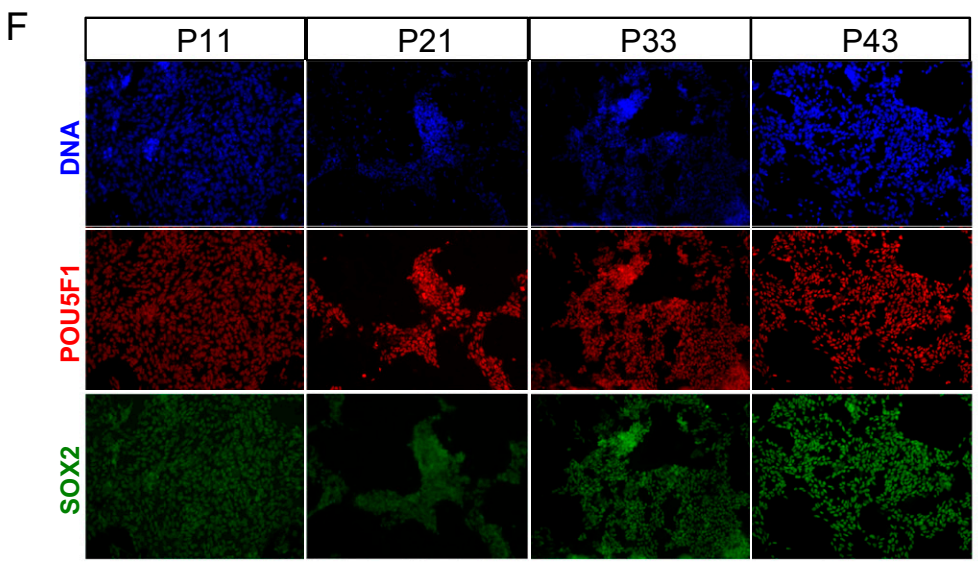


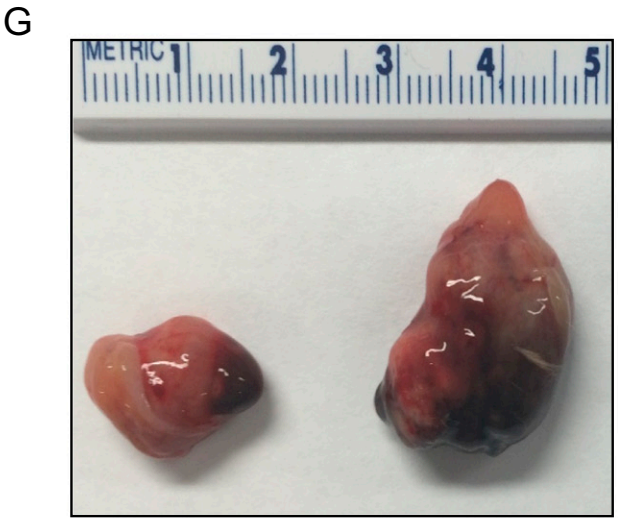




E

		CTFR-bESC A				CTFR-bESC B				Whole Blastocyst		Fibroblast	
	Gene	p13	p23	p35	p45	p12	p24	p35	p46	A	B	A	B
ICM	*POU5F1*	366.3	569.7	419.3	507.4	582.9	539.0	257.0	514.2	48.5	38.1	0.2	0.0
ICM	*NANOG*	17.2	26.3	20.1	27.0	29.2	46.1	11.2	37.1	0.6	4.4	0.0	0.0
ICM	*SOX2*	204.6	154.1	172.6	159.0	186.0	170.0	178.7	158.9	1.9	3.1	1.9	0.2
ICM	*LIN28B*	54.6	34.8	45.2	36.4	40.3	39.2	44.7	32.6	2.0	0.3	0.0	0.0
ICM	*DNMT3B*	149.4	117.6	149.6	106.9	141.1	134.1	72.2	118.1	29.4	21.9	0.4	0.3
ICM	*UTF1*	11.0	15.1	20.0	12.9	15.8	17.9	9.9	13.2	0.1	0.3	0.0	0.0
ICM	*SALL4*	40.4	28.7	34.3	28.7	38.4	32.6	28.7	27.2	4.5	4.7	0.0	0.0
TE	*CDX2*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	13.3	15.7	0.0	0.0
TE	*GATA2*	0.2	0.3	0.2	0.3	0.2	0.3	0.2	0.3	8.8	3.4	1.4	3.5
TE	*GATA3*	0.2	0.4	0.1	0.2	0.3	0.5	0.6	0.4	12.6	12.9	0.0	0.2
TE	*ELF3*	0.7	1.4	0.5	1.6	1.1	1.5	0.8	1.5	0.1	0.1	0.3	0.4
TE	*FGF4*	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	2.2	4.1	0.0	0.0
TE	*TFAP2A*	0.3	0.4	0.9	0.6	0.4	0.5	0.7	0.7	0.2	0.4	21.2	12.0
PE	*GATA6*	0.3	0.7	0.4	0.4	0.4	0.4	0.7	0.5	13.5	16.5	0.0	0.0
PE	*HNF4A*	0.2	0.0	0.1	0.1	0.0	0.1	0.1	0.0	1.7	1.6	0.0	0.0
PE	*PDGFRA*	1.0	1.9	2.3	2.2	0.7	1.1	1.4	2.5	14.1	20.3	65.8	49.6





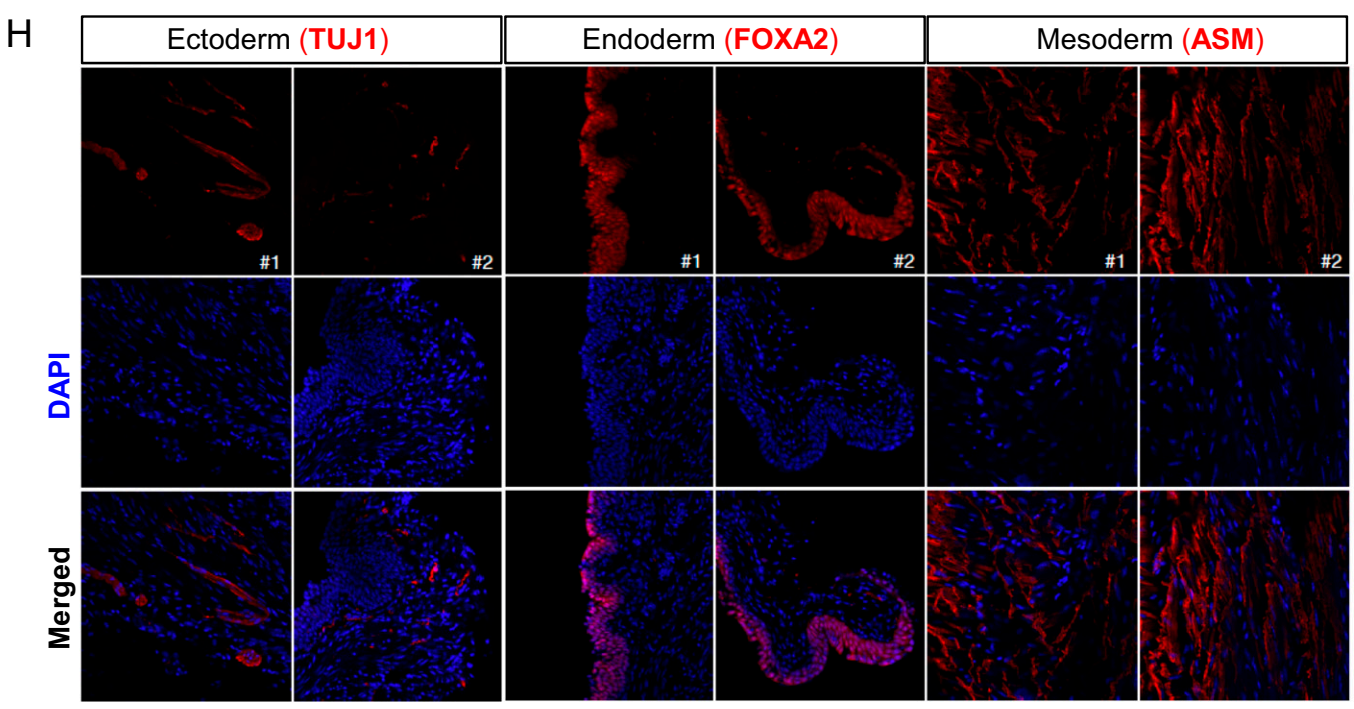
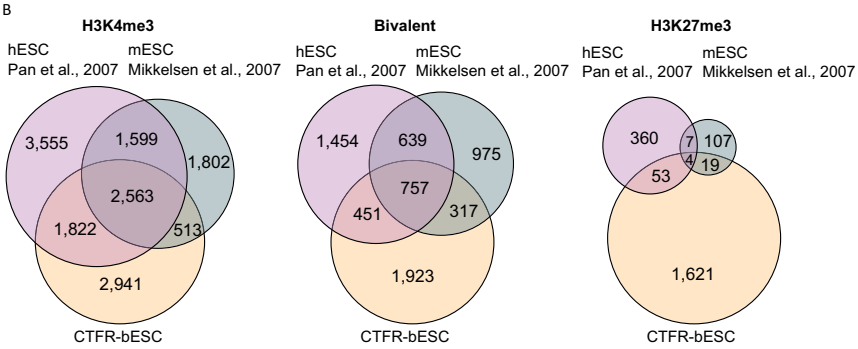


Fig. S1. CTFR-bESCs are stable and pluripotent. (*A*) Normal karyotype (2N = 60 chromosomes) observed in CTFR-bESCs at P34. (*B*) Average population-doubling time for two CTFR-bESC lines at passages P13, P23, P35, and P45. Data are shown as mean ± SEM. (*C*) Principal component analysis of RNA-seq data from two lines of CTFR-bESCs collected at different passages (CTFR-bESC A at P13, P23, P35, and P45 and CTFR-bESC B at P13, P24, P35, and P46), two whole bovine blastocysts, and two bovine fibroblast cell lines. The blue circle clusters all CTFR-bESC lines independent of the passage numbers. The red circle clusters blastocysts, and the green circle clusters fibroblast cell lines. (*D*) Heatmap showing global transcriptome analysis of CTFR-bESC A at P13, P23, P35, and P45 and CTFR-bESC B at P13, P24, P35, and P46, two whole bovine blastocysts (BL A and BL B), and two bovine fibroblast cell lines (Fib A and Fib B). (*E*) Expression analysis of ICM, TE, and PE lineage-specific markers in CTFR-bESC A at P13, P23, P35, and P45 and in CTFR-bESC B at P13, P24, P35, and P46, whole blastocysts, and fibroblasts. Transcriptome analysis was performed using RNA-seq, and RPKM values are shown. The color scale goes from red (high expression) to green (low/no expression). (*F*) IF staining for SOX2 (green) and POU5F1 (red) in CTFR-bESCs at P11, P21, P33, and P43. (*G*) Teratomas obtained from two different CTFR-bESC lines (A and B) 8 wk after injection in the leg muscle of NSG SCID mice. (*H*) IF staining for TUJ1, FOXA2, and ASM indicated that bESC-derived teratomas expressed markers of the three germ layers. (Magnification: *A*, 100×; *F* and *H*, 10×.)

A

		% Mapped reads	Uniquely mapped reads
CTFR-bESC Rep 1	Input	74.3	55,987,347
	H3K4me3	60.7	15,340,811
	H3K27me3	76.4	27,317,967
CTFR-bESC Rep 2	Input	76.3	57,840,518
	H3K4me3	60.1	16,400,918
	H3K27me3	86.2	16,015,709

B



C

CTFR-bESC-marked genes	Shared with hESC and mESC	Shared only with hESC	Shared only with mESCs	Total shared
Only H3K4me3 (n=7,839)	2,563 (32.69%)	1,822 (23.24%)	513 (6.54%)	4,898 (62.48%)
Bivalent domains (n=3,448)	757 (21.95%)	447 (12.96%)	317 (9.19%)	1,521 (44.11%)
Only H3K27me3 (n=1,697)	4 (0.23%)	53 (3.1%)	19 (1.11%)	76 (4.47%)

Fig. S2. ChIP-seq analysis and comparison of the CTFR-bESC histone methylation landscape with human and mouse ESCs. (*A*) Reads were mapped to the UMD3.1 bovine reference genome using bwa-aln. The percentage of mapped reads and the total number of uniquely mapped reads used for peak calling are shown for input, H3K4me3, and H3K27me3 samples for two biological replicates. (*B*) Venn diagrams showing the overlap between H3K4me3, H3K27me3, and bivalent genes found in mouse (1), human (2), and CTFR-bESCs. (*C*) Total numbers of genes and percentages of overlap across species for each category.

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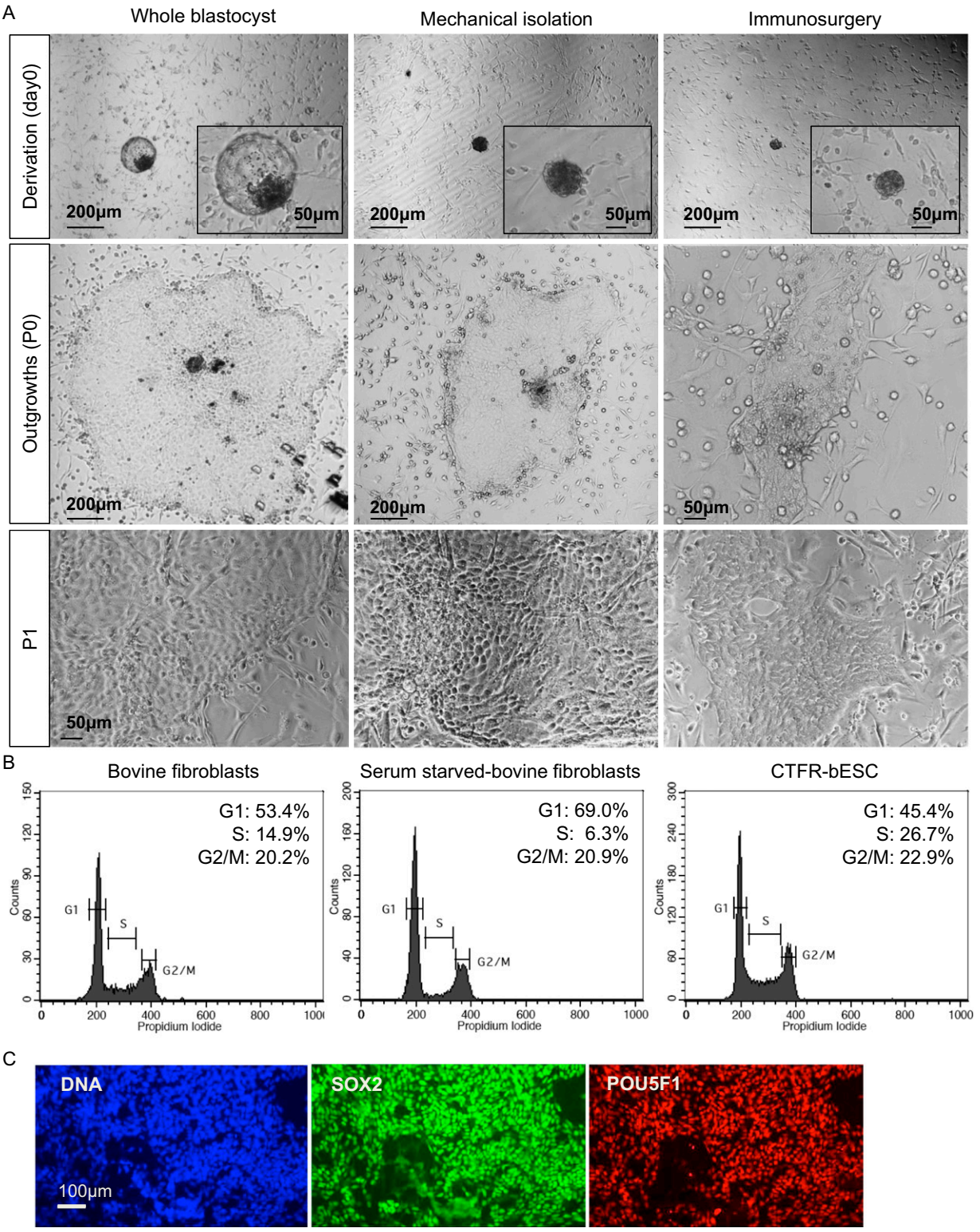

Fig. S3. CTFR-bESCs can be derived utilizing different plating methods and can be used to produce NT embryos. (*A*) Representative images showing the morphology of explants at the time of seeding (day 0), outgrowths (day 7), and CTFR-bESC P1 (day 14) for three different plating methods. (*B*) Flow cytometric analysis of the cell cycle in bovine fibroblasts, serum-starved bovine fibroblasts, and CTFR-bESCs. (*C*) IF analysis of SOX2 and POU5F1 expression in CTFR-bESCs derived from CTFR-bESC NT embryos.

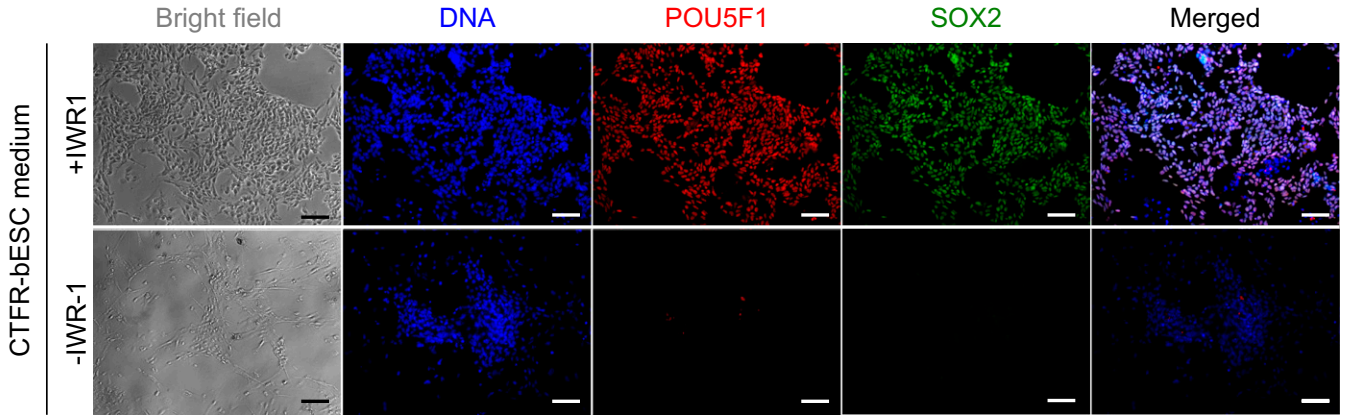

Fig. S4. Inhibition of the canonical Wnt-signaling pathway is a requirement for self-renewal of pluripotent CTFR-bESCs. Immunostaining of POU5F1 and SOX2 in bESCs grown in medium supplemented with or devoid of IWR1 (+IWR1 and –IWR1, respectively). Cells were grown for four passages in the two conditions before immunostaining analysis. (Scale bars, 100 μm.)