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Supplemental Information

Nanog Is Dispensable for the Generation of Induced Pluripotent Stem Cells

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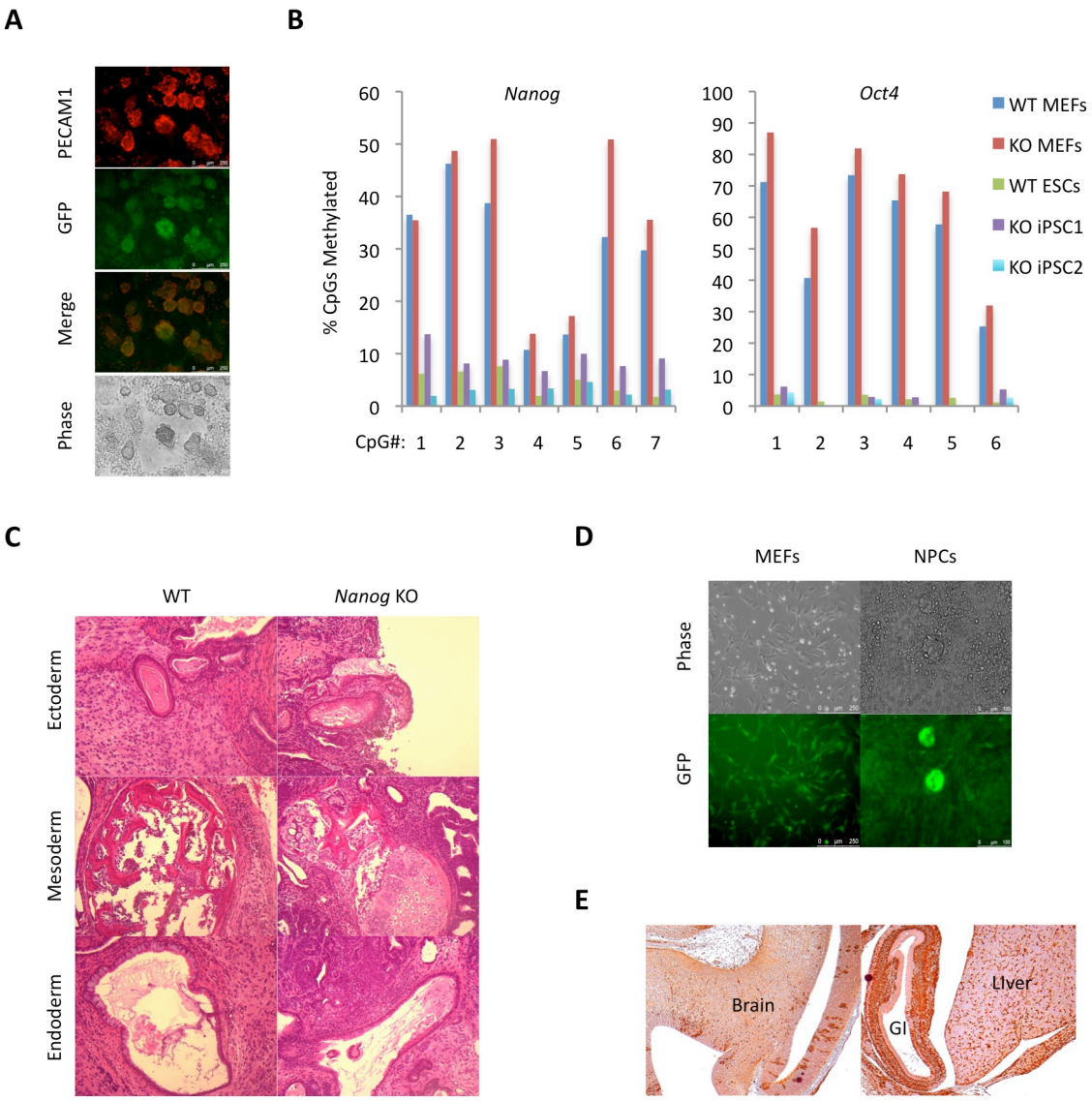

Figure S1, related to Figure 1. Characterization of *Nanog*$^{-/-}$ iPSCs.
(A) Live staining of a mixed culture of *Nanog*$^{-/-}$ (GFP+) and wild-type (GFP–) iPSCs for PECAM1. (B) Methylation analysis of the *Nanog* and *Oct4* promoters by bisulphite treatment followed by pyrosequencing of DNA from the indicated populations. (C) Tissue sections from teratomas derived from wild-type or *Nanog*$^{-/-}$ iPSCs were stained for H&E and analyzed by microscopy. Regions of ectoderm-, mesoderm-, and endoderm-derived tissue could be readily identified as indicated. (D) Fluorescent images of MEFs and NPCs generated from *Nanog*$^{-/-}$ iPSC chimeras. (E) Immunohistochemistry for GFP expression on tissue sections from a representative *Nanog*$^{-/-}$ iPSC chimera.

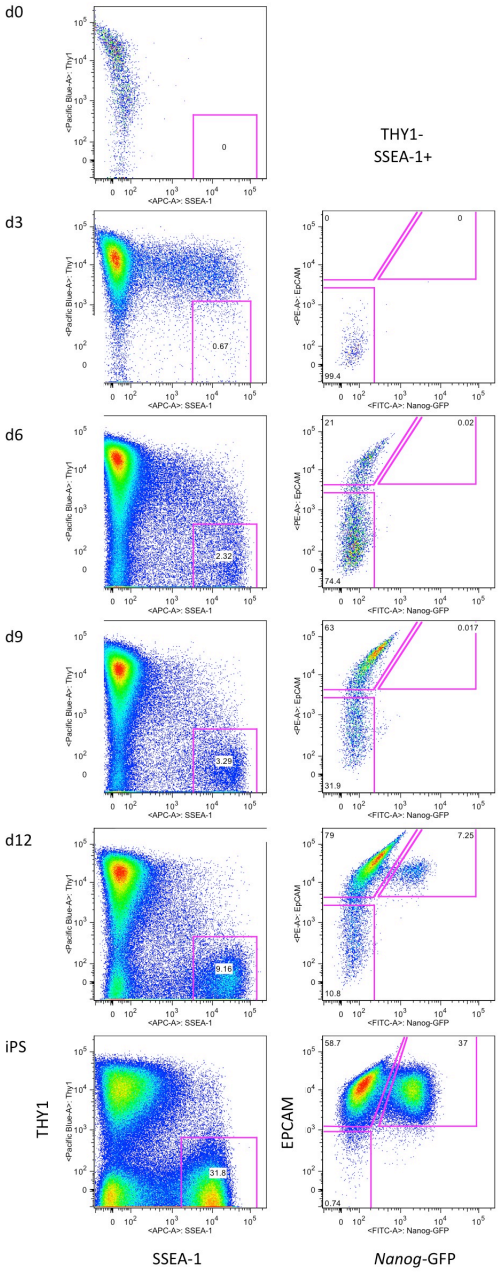

Figure S2, related to Figure 2. *Nanog-GFP* expression during reprogramming.
MEFs were derived from mice carrying a *Nanog*-GFP reporter and the tetOP-OKSM/R26-M2rtTA reprogrammable alleles (*Nanog*-GFP^{het}, *Col-OKSM*^{het}, *rtTA*^{het}) (top panel). Cells were reprogrammed by the addition of dox for 12 days. Intermediates at d3, d6, d9, d12, and iPSCs were analyzed by flow cytometry for Thy1, SSEA-1, EpCAM, and *Nanog*-GFP expression. Plots on the right were gated on Thy1^{-}SSEA-1^{+} cells.

Supplemental Experimental Procedures

Mice. All procedures, including housing and maintenance of animals, were performed according to a mouse protocol approved by the MGH Subcommittee on Research Animal Care.

Generation of *Nanog*$^{-/-}$ MEFs. *Nanog*$^{-/-}$ ESCs were generated as previously described [S1], expressing CAG-GFP and neomycin resistance under the control of the *Nanog* promoter. MEFs were generated from *Nanog*$^{-/-}$ ESCs as previously described [S2]. Briefly, ESCs were injected into E3.5 wild-type blastocysts to generate chimeras, which were transplanted into the uterus of a pseudo-pregnant female mouse. Embryos were harvested at mid-gestation to isolate MEFs (see below).

Reprogramming and cell culture. MEFs were tranduced with lentiviral STEMMCA and rtTA as previously described [S3] and passaged at least once prior to reprogramming. Reprogramming experiments were performed in gelatin-coated plates in ESC medium [KO-DMEM (Invitrogen) with L-glutamine, penicillin/streptomycin, nonessential amino acids, β-mercaptoethanol, 1,000 U/mL LIF, and 15% FBS (Invitrogen)] supplemented with 1 ug/mL of doxycycline (dox) and 50 ug/mL of ascorbic acid (AA) except where indicated. 2 inhibitors (2i) GSK-3βi (CHIR99201; Stemgent) and MEKi (PD0325901; Stemgent) were added to the

ESC medium where indicated. Dox, AA, and 2i were withdrawn at d12 of reprogramming, followed by 4 to 6 additional days of culture in ESC medium. To assay reprogramming efficiency, colonies were analyzed for GFP fluorescence under an inverted fluorescent microscope, followed by alkaline phosphatase staining (Vector Laboratories). Established iPSCs were cultured in either ESC medium or 2i/LIF medium [NDiff 227 (StemCells) supplemented with 2i] [S4]. Neomycin (G-418) was added only for the maintenance of *Nanog*$^{-/-}$ iPSCs.

Flow cytometry and cell sorting. MEFs, iPSCs, or reprogramming intermediates at the indicated time after dox induction were dissociated with trypsin (or EDTA for PECAM1 stains only). Cells were then stained with eFluor 450-conjugated anti-mouse Thy1.2 (53-2.1; eBioscience), AlexaFluor 647-conjugated anti-mouse/human SSEA-1 (MC-480; BioLegend), and phycoerythrin (PE)-conjugated anti-mouse-EpCAM (G8.8; eBioscience), anti-mouse-PECAM1 (390; eBioscience), or an isotype-matched control antibody (eBR2a; eBioscience). DAPI was used for dead cell exclusion. Cell sorting was performed on a FACSAria-II (BD) for GFP+ or GFP– Thy1^{-} SSEA-1^{+} iPSCs. Flow cytometry was performed on a LSR-II (BD).

Teratoma assay and blastocyst injections. FACS-sorted GFP+ and GFP– iPSCs were expanded in culture (with neomycin selection for GFP+ cultures only). GFP expression or absence was verified by fluorescent microscopy. Cells were dissociated with trypsin. To generate teratomas, $2x10^6$ iPSCs were injected

subcutaneously into each flank of a C.B-17 SCID mouse (Taconic). Mice were sacrificed 3 weeks later. Teratomas were isolated and processed for histological analysis and hematoxylin and eosin (H&E) staining. Blastocyst injections were performed by the Harvard University Genome Modification Facility. iPSCs were injected into E3.5 B6xBDF1 blastocysts and then transplanted to the uterus of a pseudo-pregnant female recipient. Embryos were harvested at E13.5 and analyzed for GFP chimerism by fluorescent microscopy. Some embryos were fixed and processed for histological analysis.

Generation of MEFs and NPCs. MEFs were isolated by trypsin digestion of E13.5 chimeric embryos followed by culture in fibroblast medium [DMEM (Invitrogen) with 10% FBS, L-glutamine, penicillin/streptomycin, nonessential amino acids, and β-mercaptoethanol]. MEFs were also produced from embryos derived from *Nanog*-GFP reporter mice [S5] crossed with *Col-OKSM* and *Rosa26-rtTA* mice [S6] (*Nanog*-GFP^{het}, *Col-OKSM*^{het}, *rtTA*^{het}). NPCs were isolated by collagenase IV digestion of the brains E13.5 chimeric embryos followed by culture in NPC medium (NDiff 227 supplemented with FGF and EGF).

Immunofluorescence. A mixed culture of iPSCs (GFP+ and GFP– together) were fixed with 10% formulin and permeabilized with 0.5% Triton X-100. The cells were then stained with primary antibodies to mouse OCT4 (Santa Cruz, sc-8628), mouse SOX2 (Santa Cruz, sc-17320), and mouse NANOG (Abcam, ab21603), followed by

staining with the respective secondary antibodies conjugated to AlexaFluor 546 (Invitrogen). Nuclei were counterstained with DAPI (Invitrogen). For live staining, cells in culture were stained with PE-conjugated anti-mouse PECAM1. Cells were imaged using a Leica DMI4000B inverted fluorescence microscope equipped with a Leica DFC350FX camera. Images were processed and analyzed using Adobe Photoshop software.

Immunohistochemistry. Tissue sections from chimeric embryos were stained with either H&E or anti-GFP (Living colors, JL-8; Clontech). Specific antibody staining was revealed using the M.O.M. kit, Vectastain ABC kit, and the DAB substrate (all Vector Labs). Slides were then counterstained with hematoxylin.

Microarray analysis. RNA was isolated and extracted using the RNeasy Micro Kit (Qiagen) and treated with DNase. Microarrays were performed by the Partners Center for Personalized Genetic Medicine microarray facility using the GeneChip Mouse Gene 2.0 ST Array (Affymetrix). Hierarchical clustering analysis was performed using Expander (EXPression Analyzer and DisplayER) software. All microarray data will be available from the GEO repository.

DNA methylation analysis. Frozen cell pellets were sent to EpigenDx (Hopkinton, MA) for DNA isolation, bisulphite treatment, and pyrosequencing. For the *Nanog*

promoter region, CpGs 1-7 correspond to positions –541, –502, –491, –434, –412, –302, and –280 base pairs from the transcription start site, respectively. For the *Oct4* promoter region, CpGs 1-6 correspond to positions –165, –140, –127, –103, –99, and –47 base pairs from the transcription start site, respectively.

Supplemental References

S1. Silva, J., Nichols, J., Theunissen, T., Guo, G., van Oosten, A., Barrandon, O., Wray, J., Yamanaka, S., Chambers, I., and Smith, A. (2009). Nanog is the gateway to the pluripotent ground state. Cell *138*, 722-737.

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