Supporting Information

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SI Methods

Generation of Transgenic NG4 ESC Lines. NG4 ESCs have been described (1). For ectopic Nanog expression, a Flag and biotin-tagged doxycycline-inducible Nanog transgene (^{FLbio}*Nanog*) was introduced into NG4 cells by lentiviral infection using a pTRIPZ vector. Positive clones were established by puromycin (1 μg/mL) selection, and expression of ^{FLbio}*Nanog* upon Doxycycline (Dox) treatment (0, 0.625, 1.25, or 2.5 μg/mL) was confirmed by RT-quantitative PCR (qPCR). The same Dox concentrations were used throughout the study. For knockdown studies, ESCs were infected with pLKO lentiviruses expressing no shRNA (shEmpty) or shRNA against Nanog (shNanog) or NuRD complex proteins followed by selection with puromycin (1 μg/mL) for 48 h. The pLKO.1 lentiviral vector contains a puromycin-IRES-mCherry "pim" expression cassette.

In Vitro Differentiation. Differentiation of ESCs to embryoid bodies (EBs) was performed as described in our previous study (2). Briefly, shRNA virus transduced ESCs described above were cultured in suspension on low attachment dishes in standard ES medium over a 10-d period in the absence of LIF and in the presence of puromycin. The area of EBs was calculated by using ImageJ software.

Validation of Anti-Zfp281 Antibody for Immunoprecipitation, Coimmunoprecipitation (Co-IP), and Western Blot Analyses. We have used an anti-Zfp281 antibody for both IP/co-IP and affinity purification. The quality of this antibody has been validated (Fig. S2 *A* and *B*). This antibody is commercially available from Abcam (ab101318). Other commercially available antibodies used in this study are as follows: anti-Nanog (AB5731, Millipore; A300-397A, Bethyl), anti-Chd4 (A301-081A, Bethyl), anti-Mta1/2 (A300-911A, Bethyl), anti-HDAC2 (A300-705A, Bethyl), and anti-β-Actin (A5441, Sigma).

Generation of iPSCs from *Oct4*-GFP Mouse Embryonic Fibroblasts (MEFs). For lentivirus production, STEMCCA plasmid (3) was cotransfected with packaging vectors into HEK293T cells. For retroviral production Plat-E cells were transfected with an LMP-shRNA scramble vector and LMP-shRNA against Zfp281. The viral supernatants were harvested after 48 h and concentrated by using Amicon Ultra (Millipore) centrifuge tubes. Reprogramming was performed according to a published procedure (3) with minor changes. Briefly, STEMCCA lentiviral supernatants containing the four reprogramming factors (Oct4, Sox2, Klf4, and cMyc; OSKM) and retroviral LMP-shRNA supernatants were mixed before infection. Infected cells were selected with puromycin (1.5 μg/mL) at day five after transduction, and selection was maintained for 15 d. iPSC colonies were scored 21 d after transduction by counting GFP-positive colonies under fluorescence microscope or by staining for alkaline phosphatase (AP) activity using a commercial kit (Sigma). Quantitation of GFP(+) cells during reprogramming was also performed by flow cytometry analysis.

Generation of iPSCs from Neural Stem (NS) Cell Reprogrammed PreiPSCs. *Nanog*$^{+/+}$ or *Nanog*$^{-/-}$ NS cells were reprogrammed to preiPSCs by Oct4, Klf4, and c-Myc (4, 5). These preiPSCs were infected with lentiviruses (pTRIPZ) or retroviruses (LMP or pMx) expressing shZfp281 (V2THS_42594, Open Biosystems; Table S1), scramble shRNA (shSCR), or Nanog cDNA (PiggyBac or PB-Nanog, pMx-Nanog), followed by selection with puromycin (2 μg/mL) for at least 10 d. Transduced preiPSC cells (1×10^5) were seeded on a six-well plate in serum/LIF medium. After 4 d, medium was switched to serum-free N2B27 supplemented medium with LIF and 2i inhibitors, CHIR99021 (3 μM; STEMGENT) and PD025901 (1 μM; STEMGENT). *Oct4*-GFP positive colonies from reprogrammed *Nanog*$^{+/+}$ preiPSCs or AP positively stained colonies from both *Nanog*$^{+/+}$ and *Nanog*$^{-/-}$ preiPSCs were scored at day 10 after 2i/LIF treatment.

Heterokaryon-Based Reprogramming. Experimental heterokaryons were generated by fusing mouse ESCs and human B lymphocytes according to a published protocol (6). *Zfp281*$^{+/+}$ and *Zfp281*$^{-/-}$ ESCs (2) were used for fusion with human B cells as described (6). Reprogramming of human somatic cells was monitored by quantitative real-time PCR analyses of human-specific gene expression. The sequences of human gene-specific primers are provided in Table S1.

Flow Cytometry. For flow cytometry analyses, single-cell suspensions were evaluated on an LSRII Flow Cytometer System (BD Biosciences). Data were analyzed with FlowJo software.

Chromatin Immunoprecipitation Coupled with Quantitative Real-Time PCR (ChIP-qPCR). ChIP assays were performed as described (7). Briefly, cells were cross-linked with 1% (wt/vol) formaldehyde for 5 min at room temperature, and formaldehyde was inactivated by the addition of 125 mM glycine. Chromatin extracts containing DNA fragments were immunoprecipitated by using anti-Nanog (Bethyl), anti-Zfp281 (Fig. S2), anti-Mta1/2 (Bethyl), or anti-HDAC2 (Bethyl) antibodies. Immunoprecipitated DNA was analyzed by real-time PCR as described (2), and the primer sequences are provided in Table S1. Measurements were performed in triplicate, and error bars denote SDs.

1. Schaniel C, et al. (2009) Smarcc1/Baf155 couples self-renewal gene repression with changes in chromatin structure in mouse embryonic stem cells. *Stem Cells* 27: 2979–2991.
2. Fidalgo M, et al. (2011) Zfp281 functions as a transcriptional repressor for pluripotency of mouse embryonic stem cells. *Stem Cells* 29:1705–1716.
3. Sommer CA, et al. (2009) Induced pluripotent stem cell generation using a single lentiviral stem cell cassette. *Stem Cells* 27:543–549.
4. Silva J, et al. (2008) Promotion of reprogramming to ground state pluripotency by signal inhibition. *PLoS Biol* 6:e253.
5. Silva J, et al. (2009) Nanog is the gateway to the pluripotent ground state. *Cell* 138: 722–737.
6. Pereira CF, Fisher AG (2009) Heterokaryon-based reprogramming for pluripotency. *Curr Protoc Stem Cell Biol*, Chapter 4:Unit 4B.1.
7. Lee TI, Johnstone SE, Young RA (2006) Chromatin immunoprecipitation and microarray-based analysis of protein location. *Nat Protoc* 1:729–748.

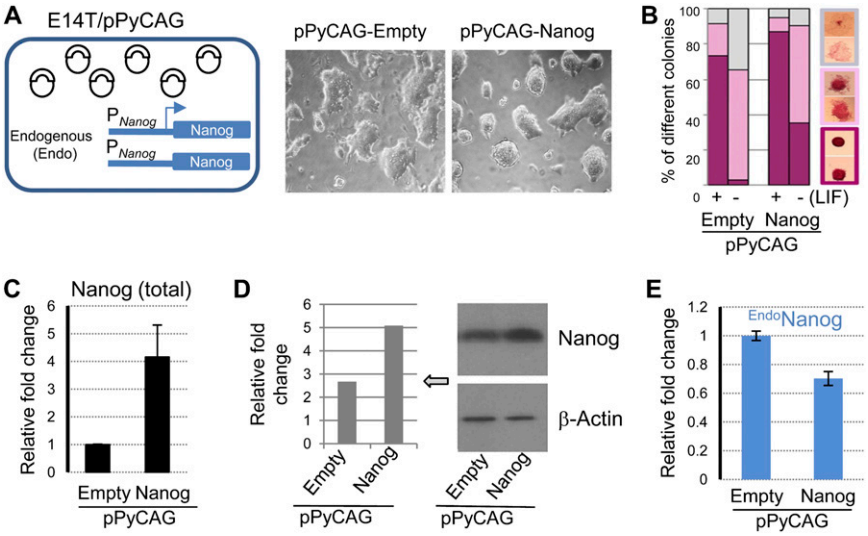

Fig. S1. Nanog autorepression in E14T cells. (*A*) Schematic representation of an episomal system for enforced *Nanog* expression in E14T ESCs (*Left*) and morphology of established E14T cell lines expressing empty vector or pPyCAG-Nanog (*Center* and *Right*). (*B*) Colony formation assays show that enforced *Nanog* expression sustains LIF-independent self-renewal. Colonies stained for AP were scored in three categories: uniformly undifferentiated (purple), partially differentiated (pink), and fully differentiated (gray) as indicated on the right. (*C*) Total *Nanog* transcripts analyzed by reverse transcription-quantitative PCR (RT-qPCR). Error bars represent SD (n = 3). (*D*) Total Nanog protein expression by Western blotting. Quantitation of protein expression was performed with ImageJ software, and results are presented on *Left*. β-Actin was used as a loading control. (*E*) RT-qPCR analyses of endogenous *Nanog* (^{Endo}*Nanog*) transcripts. Error bars represent SD (n = 3).

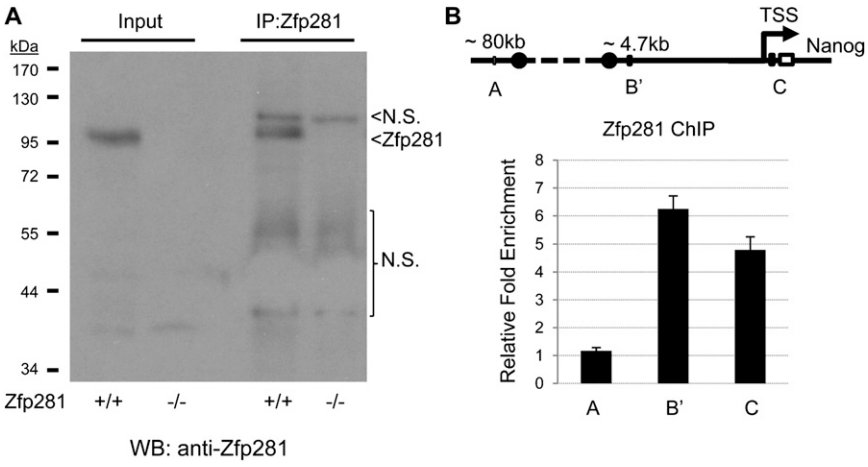

Fig. S2. Validation of Zfp281 antibody. (*A*) The Zfp281 antibody is specific for both Western blot and immunoprecipitation (IP) detection of Zfp281 protein. Zfp281 is detected by Western blot after IP of nuclear extracts from *Zfp281*$^{+/+}$ ESCs. Note that the specific band detected by Western blot disappears in the nuclear extracts from *Zfp281*$^{-/-}$ ESCs, confirming the specificity of the antibody. N.S., nonspecific signals enriched in both *Zfp281*$^{+/+}$ and *Zfp281*$^{-/-}$ ESCs during IP. (*B*) Relative enrichment of Zfp281 in the genomic loci of *Nanog* using chromatin from *Zfp281*$^{+/+}$ ESCs. Illustration of the upstream regulatory regions of the *Nanog* gene is shown (*Upper*), and primers are listed in Table S1.

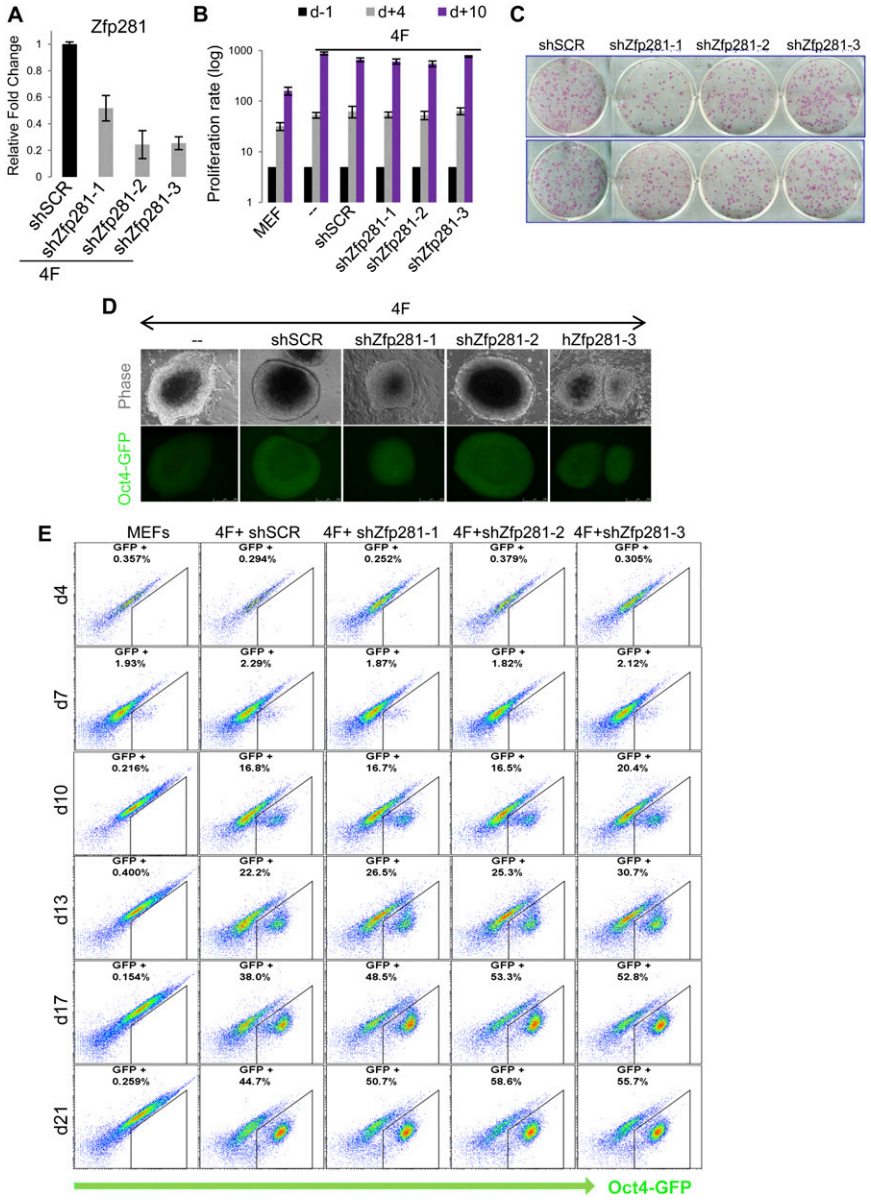

Fig. S3. Down-regulation of Zfp281 enhances the efficiency of somatic cell reprogramming. (*A*) RT-qPCR analyses of *Zfp281* expression after knockdown with three independent shRNAs targeting Zfp281. MEFs were transduced with STEMCCA lentivirus expressing four reprogramming factors (4F) (Oct4, Sox2, Klf4, and c-Myc) and LMP virus expressing a scrambled shRNA sequence (shSCR) or three independent shRNAs against Zfp281 and grown for 4 d. The data were normalized to *Gapdh*, and error bars represent the SD of triplicate qPCR reactions. (*B*) Mean proliferation rate of MEFs. Cells were counted at each time point as indicated. (*C*) iPSC colonies were stained for AP activity 21 d after transduction. Duplicated wells from the same experiment are shown. (*D*) Phase and GFP images of iPSC colonies 21 d after transduction. (*E*) Flow cytometry analysis of *Oct4*-GFP cells during iPSC reprogramming at indicated days after virus infection.

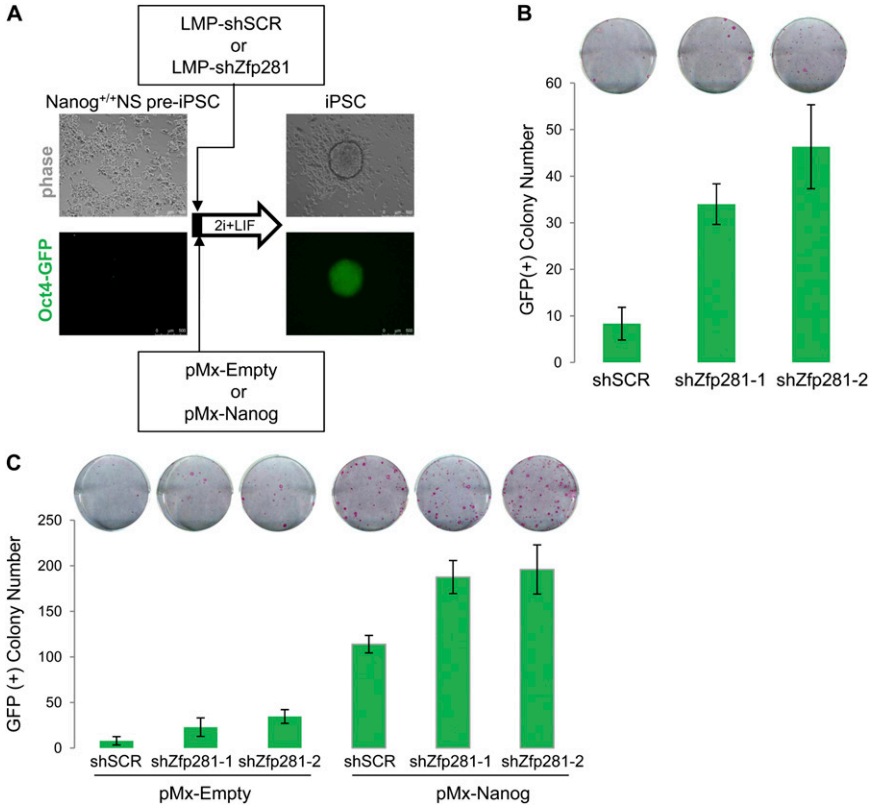

Fig. S4. Loss of Zfp281 facilitates somatic cell reprogramming through *Nanog* regulation. (*A*) Summary of the procedure for iPSC generation using *Nanog*$^{+/+}$ preiPSCs. These preiPSCs harbor an *Oct4*-GFP transgene that can be reactivated during reprogramming. (*B*) Zfp281 knockdown promotes the preiPSC to iPSC transition. (*B Upper*) AP positive colonies in representative wells. (*B Lower*) GFP (+) colony numbers. (*C*) Enhanced reprogramming by combined action of Zfp281 knockdown (shZfp281) and ectopic Nanog expression (pMx-Nanog) in *Nanog*$^{+/+}$ preiPSCs. *Nanog*$^{+/+}$ preiPSCs were infected with indicated shRNAs in combination with retroviral pMx-Nanog or pMx vector alone. (*C Upper*) AP positive colonies in representative wells. (*C Lower*) GFP (+) colony numbers.

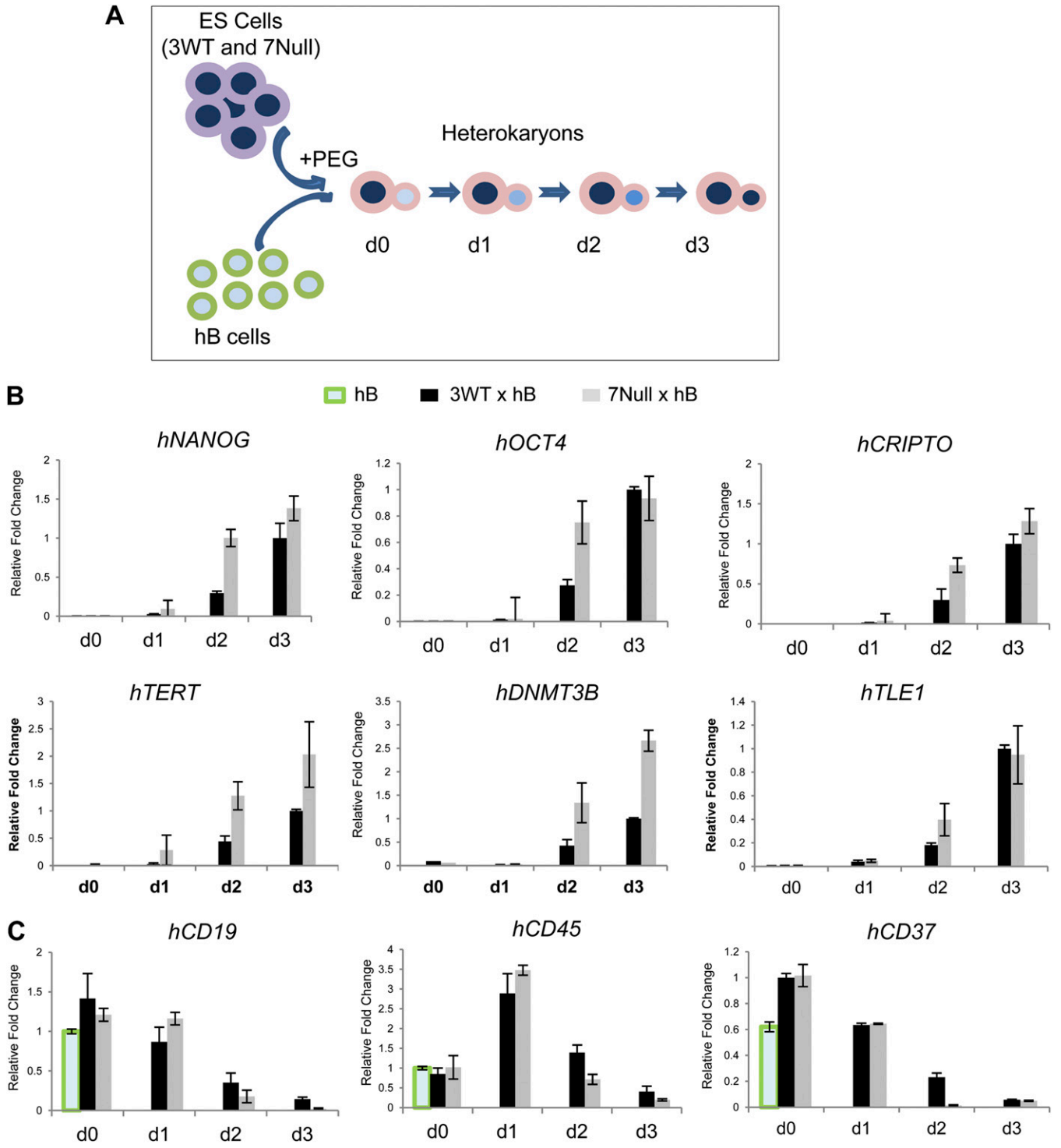

Fig. S5. Loss of Zfp281 enhances heterokaryon-based reprogramming. (*A*) Schematic representation of the strategy for generating heterokaryons between mouse ESCs and human B lymphocytes. *Zfp281*$^{+/+}$ (3WT) and *Zfp281*$^{-/-}$ (7Null) ESCs have been described (2) and were used as fusion partners for human B (hB) cells. (*B*) Enhanced reprogramming of human pluripotency gene expression in hB cells by *Zfp281*$^{-/-}$ ESCs. (*C*) Down-regulation of hB cell-specific genes during reprogramming.

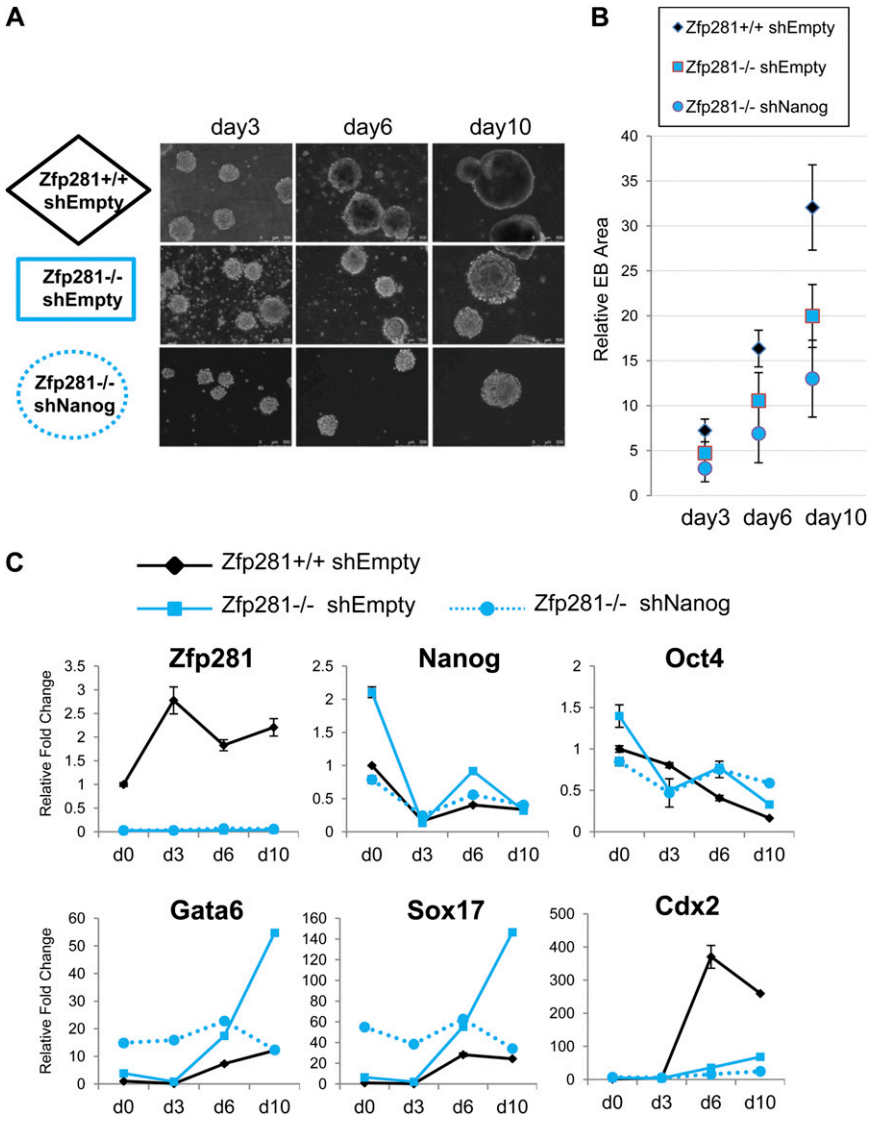

Fig. S6. Down-regulation of Nanog fails to rescue differentiation defects of *Zfp281*$^{-/-}$ ESCs during in vitro differentiation. (*A*) Representative images of EBs generated from ESCs transduced with shEmpty or shNanog in the presence or absence of Zfp281. (*B*) Analysis of the relative EB area during the time course of EB differentiation. Note that down-regulation of Nanog fails to rescue the smaller EB phenotype of *Zfp281*$^{-/-}$ ESCs. (*C*) RT-qPCR analyses of relative expression levels of *Nanog*, *Oct4*, *Gata6*, *Sox17*, and *Cdx2* during the time course of EB differentiation.

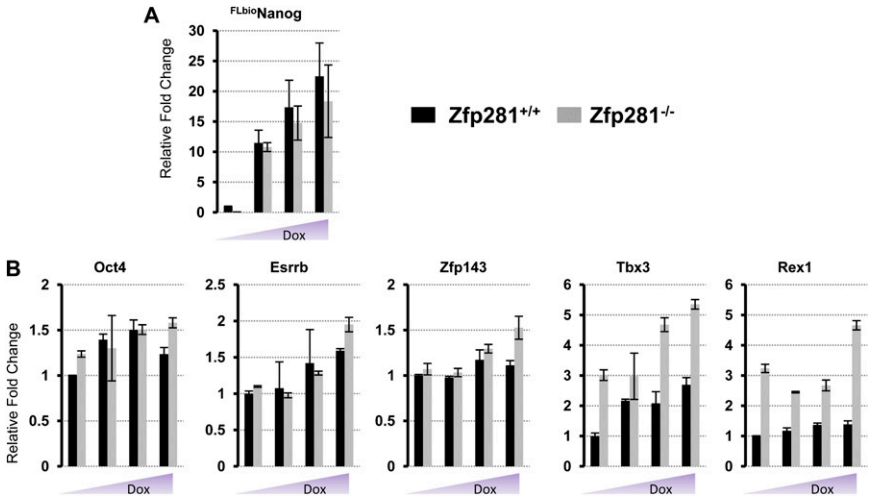

Fig. S7. Ectopic expression of Nanog promotes activation of known *Nanog* activators in the absence of Zfp281. (*A*) RT-qPCR analyses of ectopic *Nanog* (*^{FLbio}Nanog*) expression upon Dox treatment in *Zfp281$^{+/+}$* and *Zfp281$^{-/-}$* ESCs. (*B*) RT-qPCR analyses of *Oct4*, *Esrrb*, *Zfp143*, *Tbx3,* and *Rex1* expression in the same samples as described in *A*.

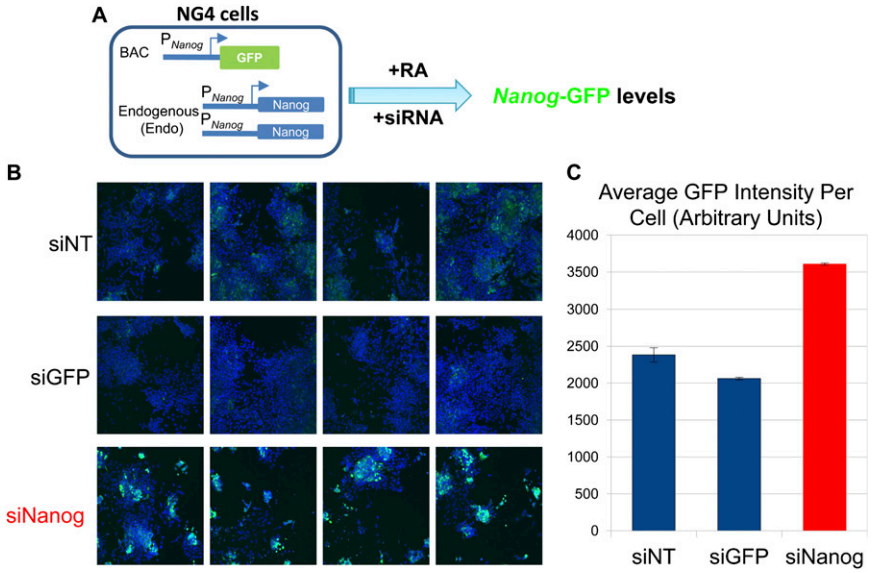

Fig. S8. Down-regulation of Nanog by siRNA causes up-regulation of *Nanog*-GFP reporter activity. (*A*) Schematic depiction of the genome-wide siRNA screening strategy. NG4 cells were reverse-transfected with 25 nM siRNA in 384-well tissue culture plates coated with 0.1% gelatin in 50 μL of total volume with standard mouse ES media containing LIF. After 1 d, media were changed to ES media without LIF plus 10 nM RA for 2 d. Cells were fixed with 2% formaledhyde in PBS for 15 min and stained with Hoechst 33342 (5 μg/mL) before confocal fluorescence imaging. (*B*) Representative high-content images of NG4 cells treated with nontargeting siRNA control (siNT), siRNA against GFP (siGFP), and siRNA against Nanog (siNanog). (*C*) Quantitation of average GFP intensity per cell. Note the increase of GFP intensity upon siNanog treatment.

Table S1. List of qPCR primers, ChIP-qPCR primers, and shRNA oligos used in this study

Primer name	Primer sequence
qPCR analysis	
Nanog F (End)	TTGCTCTTTCTGTGGGAAGG
Nanog R (end)	CCAGGAAGACCCACACTCAT
Nanog F (total)	AGGGTCTGCTACTGAGATGCTCTG
Nanog R (total)	CAACCACTGGTTTTTCTGCCACCG
Nanog F (Flagbio)	GCAAGAAGAGCATCTGTGGA
Nanog R (Flagbio)	AAAACTGCAGGCATTGATGA
Gapdh F	ACCCAGAAGACTGTGGATGG
Gapdh R	CACATTGGGGGTAGGAACAC
Gatad2b F	CCCAGCAGATGAGCGAGAT
Gatad2b R	TCTTCATAACCCTTGACACCACT
Mta2 F	TCGGGAGTTTGAGGAGGAATC
Mta2 R	CCCTTATGTGGGTGGCTGGTA
Mta3 F	CCACTTACGGATCGACAGATTG
Mta3 R	GGCGCTGCTGAGGTCATAG
Cdx2 F	CAAGGACGTGAGCATGTATCC
Cdx2 R	GTAACCACCGTAGTCCGGGTA
Esrrb F	CAGGCAAGGATGACAGACG
Esrrb R	GAGACAGCACGAAGGACTGC
Gata6 F	TTGCTCCGGTAACAGCAGTG
Gata6 R	GTGGTCGCTTGTGTAGAAGGA
Oct4 F	CTGAGGGCCAGGCAGGAGCACGAG
Oct4 R	CTGTAGGGAGGGCTTCGGGCACTT
Rex1 F	CAGTCCAGAATACCAGAGTGGAA
Rex1 R	ACTCTAGGTATCCGTCAGGGAAG
Sox17 F	CGCACGGAATTCGAACAGTA
Sox17 R	GTCAAATGTCGGGGTAGTTG
Tbx3 F	TTATTTCCAGGTCAGGAGATGGC
Tbx3 R	GGTCGTTTGAACCAAGTCCCTC
Zfp143 F	CAGGTCAAGGTGATGATGTTCTTAAAGGGT
Zfp143 R	GGCCTGCATGTCAGCTTGAGATATG
Zfp281 F	ACCTGGTGGCAGAGCTTGTGT
Zfp281 R	AGAGCAGAGCCACTGCCTATC
CD19 F (human)	GCTCAAGACGCTGGAAAGTATTATT
CD19 R (human)	GATAAGCCAAAGTCACAGCTGAGA
CD37 F (human)	GTGGCTGCACAACAACCTTATTT
CD37 R (human)	GCCTAACGGTATCGAGCGAG
CD45 F (human)	CCCCATGAACGTTACCATTTG
CD45 R (human)	GATAGTCTCCATTGTGAAAATAGGCC
CRIPTO F (human)	AGAAGTGTTCCCTGTGTAAATGCTG
CRIPTO R (human)	CACGAGGTGCTCATCCATCA
DNMT3b F (human)	GTCAAGCTACACACAGGACTTGACAG
DNMT3b R (human)	AGTTCGGACAGCTGGGCTTT
GAPDH F (human)	TCTGCTCCTCCTGTTCGACA
GAPDH R (human)	AAAAGCAGCCCTGGTGACC
NANOG F (human)	CCAACATCCTGAACCTCAGCTAC
NANOG R (human)	GCCTTCTGCGTCACACCATT
OCT4 F (human)	TCGAGAACCGAGTGAGAGGC
OCT4 R (human)	CACACTCGGACCACATCCTTC
TERT F (human)	GCCAGCATCATCAAACCCC
TERT R (human)	CTGTCAAGGTAGAGACGTGGCTC
TLE1 F (human)	TGTCTCCCAGCTCGACTGTCT
TLE1 R (human)	AAGTACTGGCTTCCCCTCCC
ChIP-qPCR analysis	
Gapdh F	AAGCTCATGAGGCACAGAATGGTC
Gapdh R	TGGGTACATGGTGACTTTCCTAGGC
Nanog-A F	GGCATTTGTGAGTATAGGGGTAGG
Nanog-A R	CCTGTTCTGTGTCAGCACACTTAG
Nanog-B′ F	GTTTTGACTGCTAACCACCCAGAG
Nanog-B′ R	GGCAGGCTTGCTACATTCCTTATC
Nanog-B′′ F	ACTCCAAGGCTAGCGATTCA
Nanog-B′′ R	AATAGGGAGGAGGGCGTCTA

Table S1. Cont.

Primer name	Primer sequence
Nanog-C F	AATGAGGTAAAGCCTCTTTTTGG
Nanog-C R	ACCATGGACATTGTAATGCAAA
shRNA sequences	
shChd4	CCTGAGAGGTTCCACAACTTA
shGatad2b	TCAACGTGTTATTGCACCAAA
shLuciferase (shLuci)	CTTACGCTGAGTACTTCGA
shMta2	CGGGAAGGATTTCAATGATAT
shMta3	CGGCAAAGATTTCAACGACAT
shNanog	GACAGTGAGGTGCATATAC
shZfp281-1	GTCATCAAACCATAACAGTA
shZfp281-2	GCCCGATAAGTAGTAATTA
shZfp281-3	CTCTAAATGCTGAAATTAAG
shZfp281 (pTRIPZ-shRNAmir)	V2THS_42594 (Open Biosystems)

F, forward primer; R, reverse primer.