Stem Cell Reports, Volume *2*

Supplemental Information

Temporal Perturbation of the Wnt Signaling Pathway in the Control of Cell Reprogramming Is Modulated by TCF1

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2) Supplemental Experimental Procedures: A detailed description of all the experimental procedures used in this work.

3) Supplemental Tables
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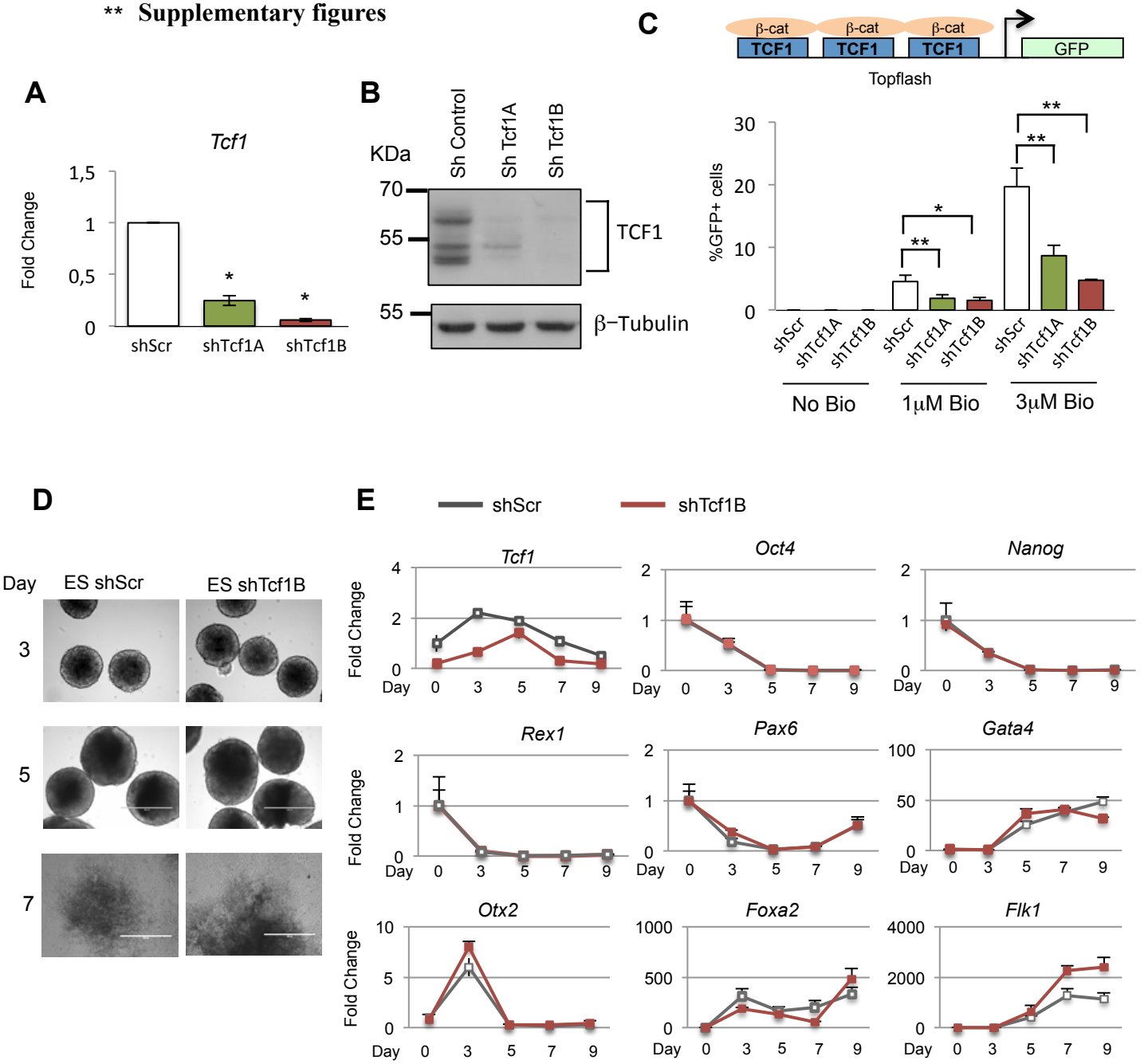
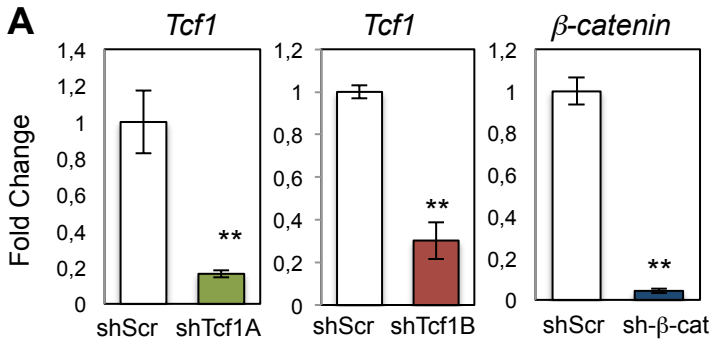
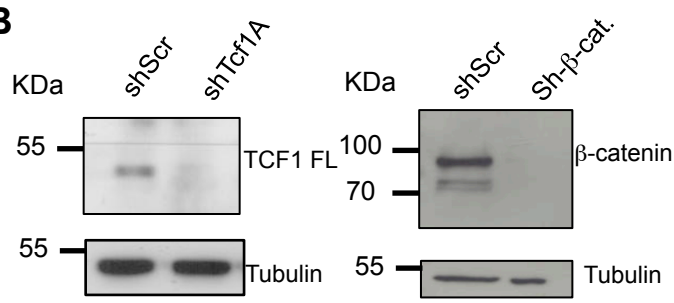
** **Supplementary figures**



Supplementary Figure S1. Tcf1 knock-down in mouse ESCs.
ESCs were infected with pLKO lentivirus carrying scrambled (shScr) or *Tcf1* silencing (shTcf1A and shTcf1B). Infected cells were selected using hygromycin. **(A)** Quantitative PCR analysis; (n = 3 independent experiments). **(B)** Representative Western blot (as indicated). **(C)** shScr, shTcf1A and shTcf1B ES cells were infected with 7TGP reporter and selected for stable incorporation. Cells were then treated with BIO at indicated concentrations during 24h to activate the Wnt/β-catenin pathway. Number of GFP+ cells was mesured by FACS analysis; (n=4 independent experiments) **(D)** ESCs-shScr and ESCs-shTcf1B were subjected to differentiation through aggregation into embryoid bodies. Representative phase-contrast images at 3, 5, and 7 days during embryoid body differentiation. Scale bar: 400μm. **(E)** Representative qRT-PCR experiment (out of two independent experiments) for the detection of *Tcf1*, stem-cell (*Oct-4*, *Nanog*, *Rex1*), ectoderm (*Pax6*, *Otx2*), mesoderm (*Flk1*) and endoderm (*Gata4* and *Foxa2*) marker genes in embryoid bodies at different times p.i.. The levels are normalised to *Gapdh*.
All pooled data are represented as means ± SD. The asterisks indicate statistical significance by t test analysis (* $p<0.05$; ** $p<0.01$).





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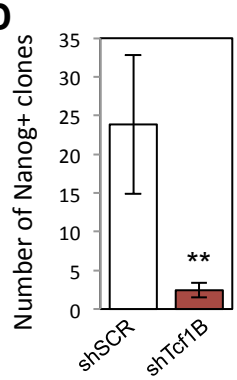
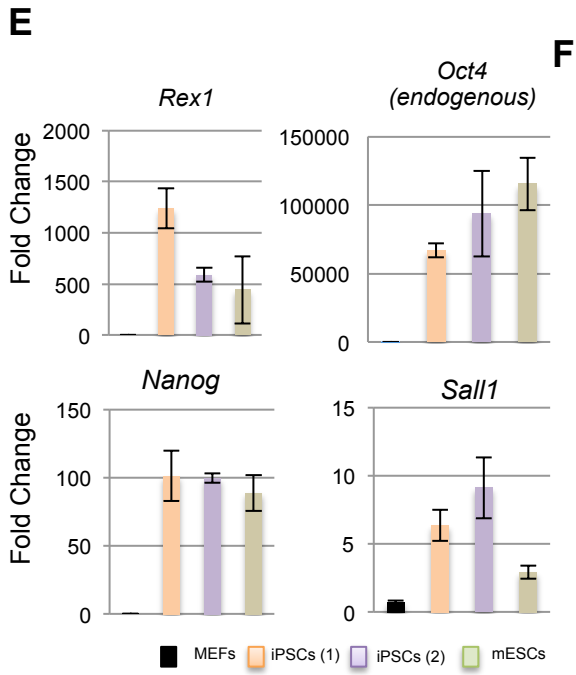
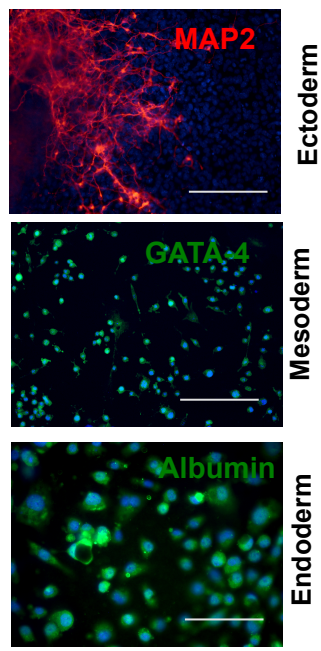
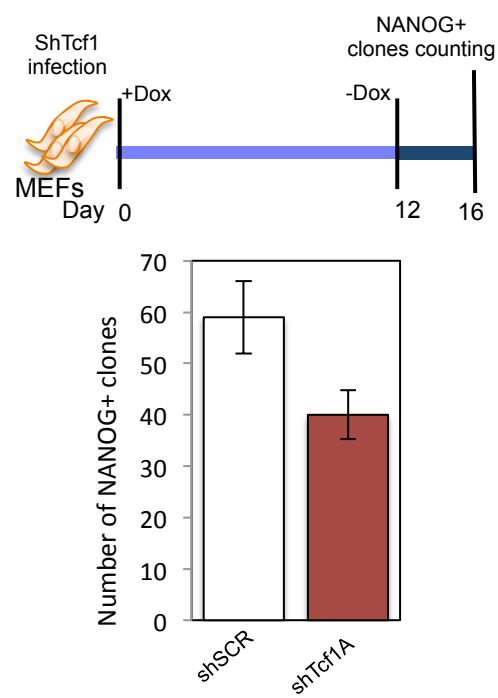
Silencing	Min %	Max %
shScr	0,0746	0,118
shTcf1A	0,014	0,046
shβcat	0,01	0,03



Infection	Number NANOG+ clones	Efficiencicy (Min-Max %)
ShScr	**24 ±9**	0,03-0066
ShTcf1B	**3 ±1**	0,004-0,008



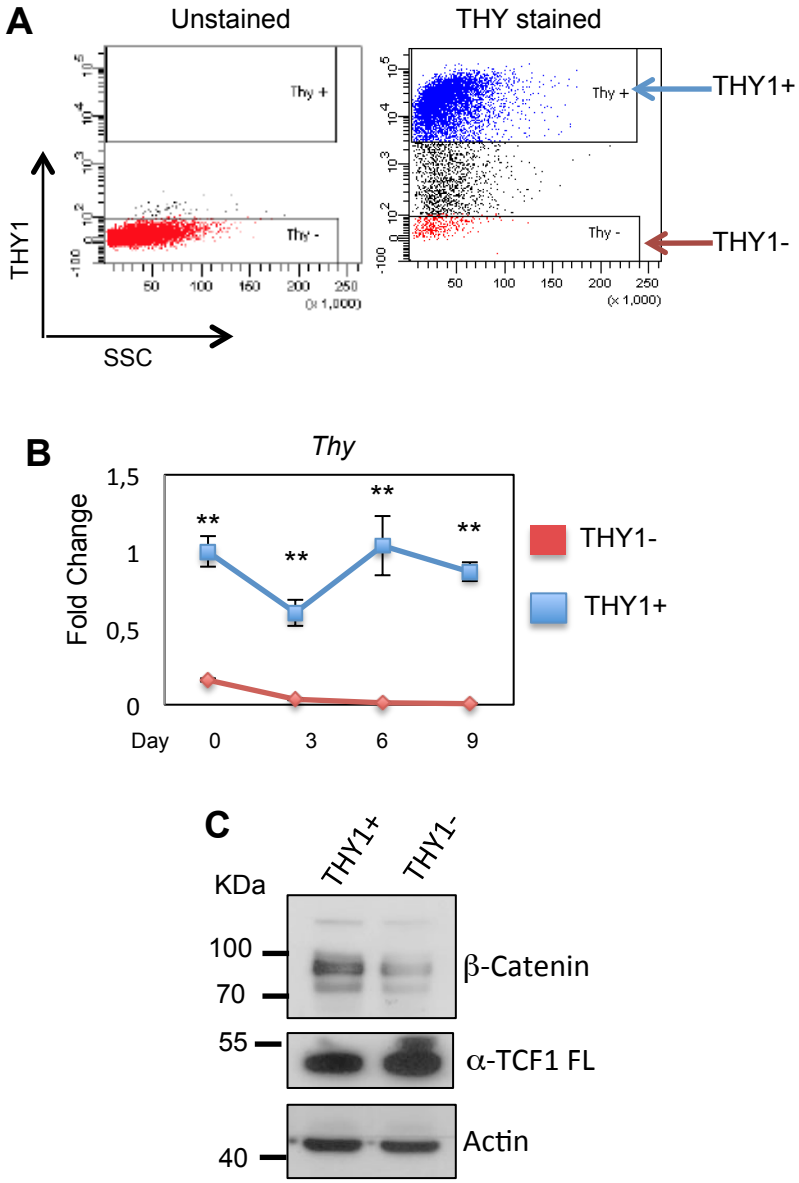




Supplementary Figure S2. Tcf1 knock-down in MEFs.

(A) Quantitative PCR analysis of *ß-catenin* and *Tcf1* expression in four-factor MEFs infected with shScr, sh-ß-catenin shTcf1A and shTcf1B, as indicated. (n = 3 independent experiments). **(B)** Representative Western blots showing ß-catenin and FLTCF1 protein levels in the cells infected with shScr, sh-ß-catenin and shTcf1A. **(C)** Table showing minimum and maximum efficiencies of reprogramming in the cells infected with shScr, sh-ß-catenin and shTcf1A. Efficiencies are calculated: Number of NANOG+ clones/ Number of plated MEFs x100. **(D)** Number of NANOG positive (NANOG$^+$) clones obtained in four-factor MEFs infected with shScr and shTcf1B according to each treatment; (n=3 independent experiments). **(E)** Quantitative RT-PCR analysis of the expression of ESC markers in MEFs, selected iPSC clones, and mouse ESCs. **(F)** Representative images of immunostaining of iPSCs differentiated into ectoderm (MAP2), mesoderm (GATA4) and endoderm (Albumin), as indicated. Scale bars: 200mm **(G)** Number of NANOG positive clones (NANOG+) after Tcf1 silencing without Hygromycin selection (n=2 independent experiments).

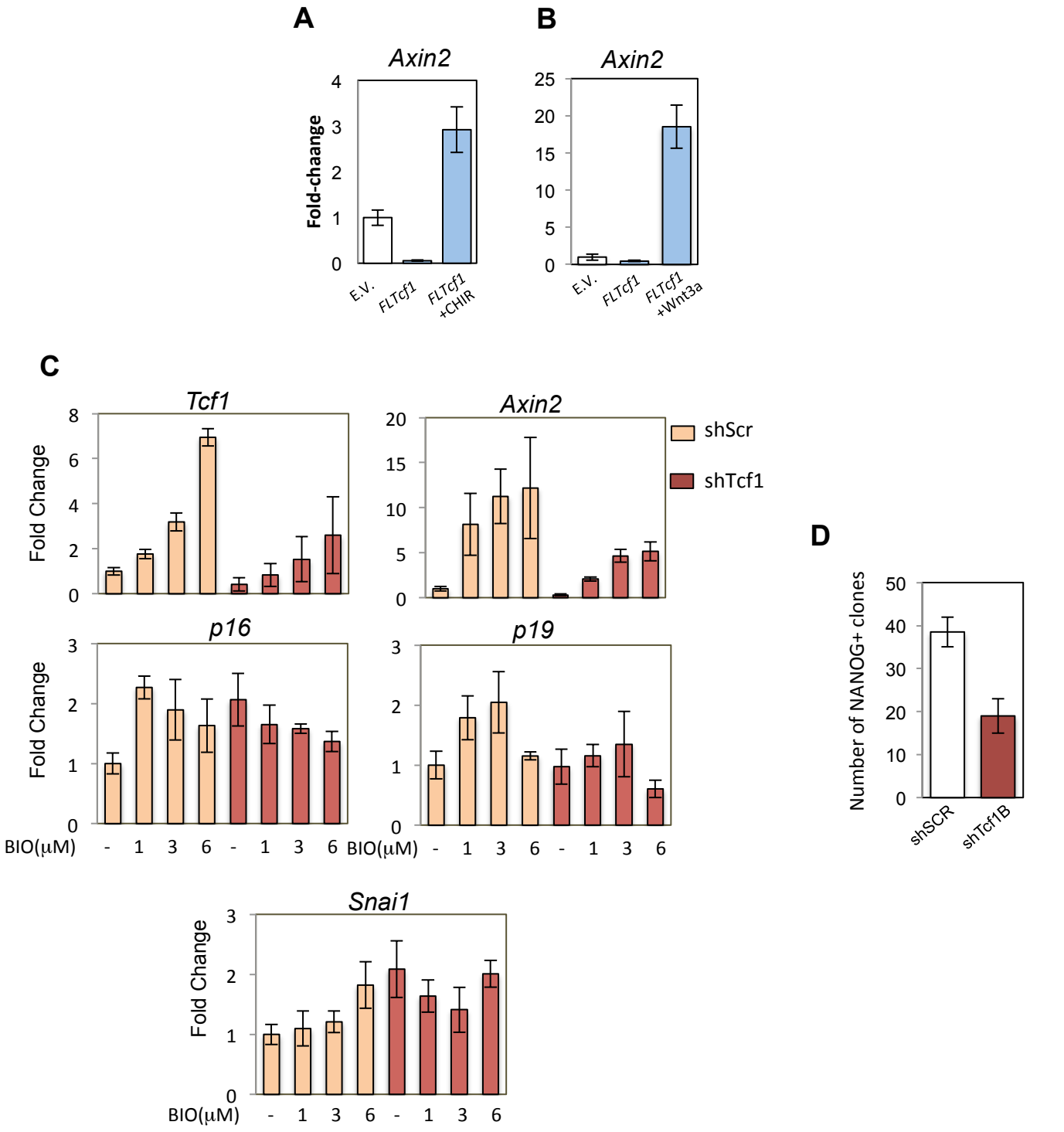
All pooled data are represented as means ± SD. The asterisks indicate statistical significance by t test analysis (* $p<0.05$; ** $p<0.01$).



Supplementary Figure S3.

(A) Four-factor MEFs were induced with doxycycline as in Figure 2A. At days 3, 6 and 9, the cells were FACS-sorted for THY1-positive (THY1^{+}; blue) and THY1-negative (THY1^{-}; red) expression (right panel), and analysed. Unstained cells were used as negative control (left panel). **(B)** Time-dependent expression of *Thy* in sorted populations (n=3 independent experiments). **(C)** Representative Western blot of total ß-catenin and TCF1 in THY1-positive and THY1-negative sorted cells 6 days after doxycycline induction.

All pooled data are represented as means ± SD. The asterisks indicate statistical significance by t test analysis (n.s. no significant; * $p<0.05$; ** $p<0.01$).



Supplementary Figure S4.

(A,B) Four-factor MEFs were infected with lentiviruses overexpressing *FLTcf1* and selected with hygromycin. Cells were then treated with Chiron or Wnt3a for 24 hours. qRT-PCR of *Axin2* levels are shown; (n=2 independent experiments). **(C)** qRT-PCR expression analysis of four-factor MEFs infected with shScr or shTcf1 and treated for 12h with increasing concentrations of Chiron as indicated (N.T.: Non-Treated); (n=2 independent experiments). **(D)** The same number of THY1-negative cells was sorted at day 6 after doxycycline induction of shScr or shTcf1B-infected four-factor MEFs, and plated on feeders. The number of NANOG+ clones was determined under each treatment; (n = 2 independent experiments).

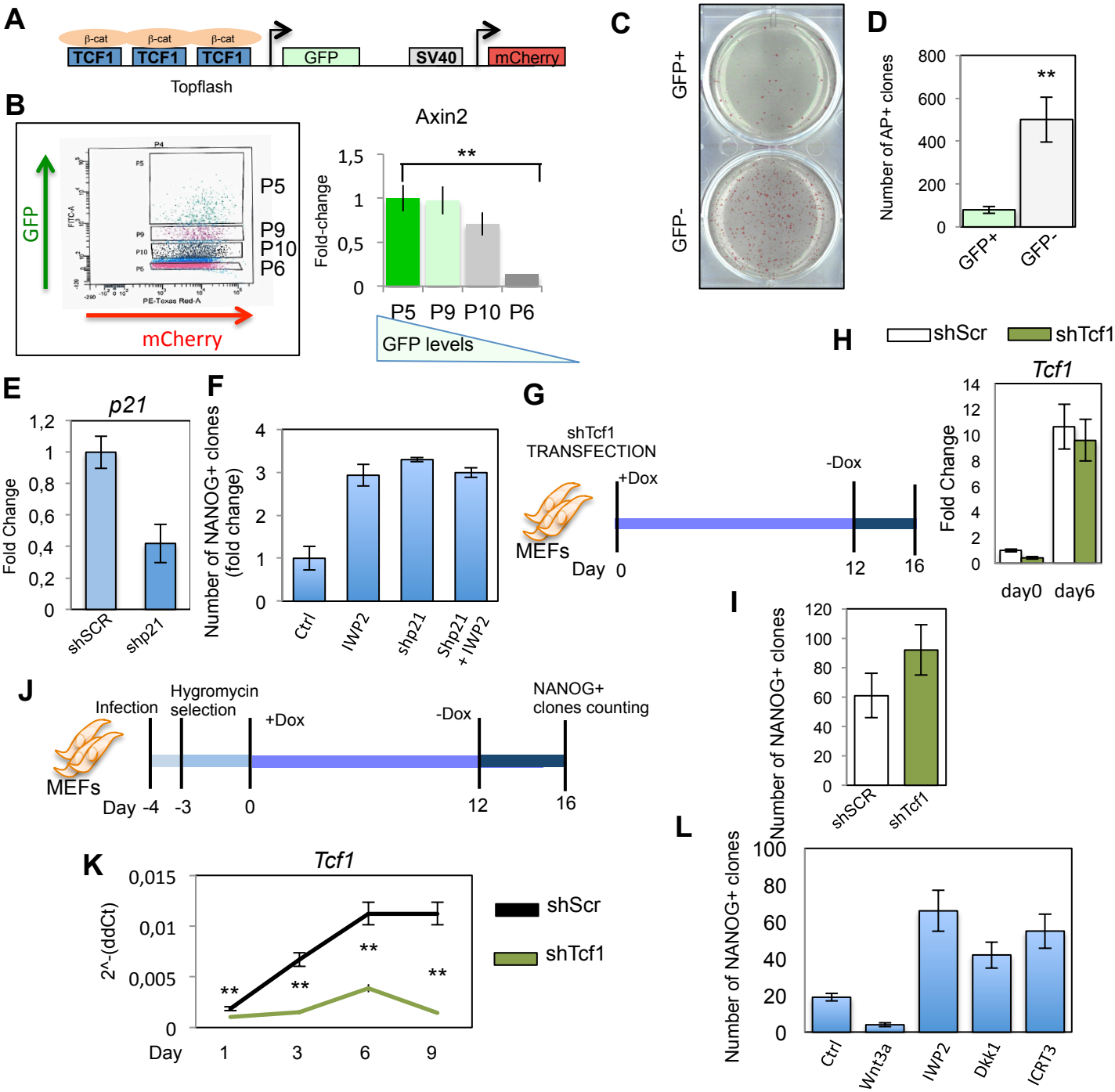


Figure S5. GFP-negative cells show an increase in the number of AP⁺ iPS colonies

(A) Scheme of 7TGC reporter. **(B)** Cells positive for Cherry expression were sorted by different levels of GFP expression (P5, P9, P10 and P6) at 6 days after doxycycline induction. qRT-PCR of *Axin2* expression is shown; (n=3). **(C)** GFP+ and GFP- cells were sorted at 6 days p.i. and replated on feeders. Doxycicline was removed at day 12. At day 16 plates were stained for Alkaline Phosphatase (AP) staining. **(D)** Quantification of the number of alkaline-phosphatase-positive (AP⁺) colonies at day 16; (n=3 independent experiments). **(E)** Quantitative PCR analysis of *p21*expression in four-factor MEFs infected with shScr and shp21 **(F)** Four-factor MEFs were infected with a lentivirus expressing a silencing for *p21* and cells were treated for 6 days with IWP2. Immunostaining against Nanog was performed, to count the number of reprogrammed clones. (n=2 independent experiments)**(G)** Experimental scheme for iPSCs generation. Four-factor MEFs were transfected with lentiviral vectors carrying shScr or shTcf1. Doxycycline was applied from 0 to 12 days and removed for the following 4 days. Immunostaining against Nanog was performed, to count the number of reprogrammed clones. **(H)** qRT-PCR of *Tcf1* levels at days 0 and 6 after transfection**. (I)** Number of NANOG positive (NANOG⁺) clones obtained according to experimental procedure indicated in (G), as indicated (n=2 independent experiments)). **(J)** Experimental procedure as in Figure 2A. **(K)** qRT-PCR levels of *Tcf1* expression in shScr and shTcf1 at 1, 3, 6 and 9 after doxycycline induction; (n=3 independent experiments). **(L)** Four-factor MEFs were pre-treated for 3 days with indicated treatments before to apply doxycycline for 12 days. The number of NANOG+ clones was determined under each treatment; (n = 2 independent experiments). All pooled data are represented as means ± SD. The asterisks indicate statistical significance by t test analysis (* $p<0.05$; ** $p<0.01$).

Supplementary Methods

Cell culture

MEFs were established from E13.5 embryos from reprogrammable mice carrying two (ho/ho) copies of the OKSM cassette and the ROSA26-M2rtTA allele (Carey et al, 2010). The embryos were isolated from the uterus and washed in phosphate-buffered saline (PBS). The head and viscera were removed, and the rest of the body was mechanically disaggregated and then incubated in 0.1% trypsin/ 0.1 mM EDTA solution for 30 min, to allow the cells to detach from the extracellular matrix and from each other. The cells were then mechanically disaggregated and plated onto a 15-cm tissue-culture dish and cultured in MEF culture medium: Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS) 10 U/ml penicillin, 10 μg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and 1× non-essential amino acids. The MEFs were kept at 37 °C under 5% CO_2.

Wnt3a (100ng/ml) and Dkk1 (100ng/ml) were obtained from R&D systems; IWP2 (2μM) was from Stemgent and ICRT3 (25μM) was purchased from Calbiochem.

Mouse ESCs (129/sv) were cultured on gelatin coated-plates in ESC medium: knock-out DMEM supplemented with 15% FBS (Hyclone), 1X non-essential amino acids, 1X GlutaMax, 1X penicillin/ streptomycin, 1X 2-mercaptoethanol and 1,000 U/ml LIF ESGRO (Chemicon). The differentiation medium for the production of embryoid bodies (EBs) consisted of this ESC culture medium without the LIF. The cells were harvested by trypsinisation, counted, and propagated in hanging drops (400 single ESCs/ 30 μl initial drop) for 2 days, before being transferred to 10 cm^2 bacterial dishes. On day 5, the embryoid bodies were transferred onto gelatinised p100 dishes always in differentiation medium.

For the iPSC differentiation the reprogrammed clones were detached from the feeder layer of MEFs and EBs were formed in ESC culture medium without LIF as described above. Furthermore, on day 5 the EBs were successively disaggregated and plated onto gelatin-coated dishes in ESC culture medium in absence of LIF, to allow them to differentiate into the three germ layers. For neural differentiation retinoic acid was added to the culture.

Constructs preparation

Short hairpins targeting ß-catenin (shß-cat), Tcf1 (shTcf1A, shTcf1B), p21 (shp21) and a short hairpin control (shScr) were cloned into the pLKO.1-Hygro lentiviral vector (Addgene plasmid #24150), following the manufacturer instructions (http://www.addgene.org/tools/protocols/plko/). The oligonucleotides cloned into the pLKO vector were purchased from Sigma-Aldrich. A list of the oligonucleotides used to generate the short hairpins is given in Supplementary Table S2. pCMV-dR8.9 dvpr (Addgene #8455) and pCMV-VSV-G (Addgene #8454) were used as lentiviral packaging constructs. The FLTcf1 (p45 Tcf-B) and ΔN-Tcf1 (p54 Tcf-E) constructs were kindly provided by Hans Clevers (Van de Wetering et al, 1996). Tcf1 constructs were cloned together with the hygromycin-resistance cassette into p1494 lentiviral vectors that were kindly provided by Luigi Naldini. The 7TGP and 7TGC lentiviral reporter were purchased from Addgene (Addgene #24305; #24304) (Fuerer and Nusse, 2010).

Virus preparation and ESC infection

For ESC infection, lentiviral particles were produced following the RNA interference Consortium (TRC) instructions for lentiviral particle production and infection in 6-well plates (http://www.broadinstitute.org/rnai/public/). Briefly 5 $\times 10^5$ HEK293T cells/well were seeded in 6-well plates. The day after plating, the cells were co-transfected with 1 µg pLKO-shTcf1A, pLKO-shTcf1B or pLKO-shScr, 750 µg pCMV-dR8.9, and 250 µg pCMV-VSV-G, using Polyfect reagent (Qiagen). The day after transfection, the HEK293T culture medium was substituted with the ESC culture medium. Then 5 $\times 10^5$ ESCs/well were plated onto gelatin-coated 6-well plates the day before transduction. The lentiviral-containing medium was harvested from HEK293T cells at 48, 72 and 96 h after transfection, filtered, and added to the ESC plates. The day after transduction, these ESCs were washed twice in PBS and hygromycin selection (50 µg/ml) was applied.

Transient transfection of MEF cells

For a transient silencing of Tcf1 during reprogramming, $1x10^6$ MEF cells were elctroporated with pLKO-shSCR and pLKO-shTcf1A using Amaxa reagent (Amaxa #VPD-1004) following manufacturer's instructions. After nucleofection, $1x10^5$ cells were plated in a 35mm dish.

Flow cytometry and immunofluorescence

For analysis and/or sorting of intermediates, cells were trypsinised, washed once in PBS, and resuspended in PBS with 5% FBS. These harvested cells were incubated with antibodies against Thy1.2.2; 0.04 μg antibody per 1 $\times 10^6$ cells (PE, eBioscience) for 20 min, washed twice in PBS plus 5% FBS, and sorted or analysed as indicated. Unstained cells were used as the negative staining control.

For immunocytochemistry, the cells were fixed with 4% paraformaldehyde for 20 min at room temperature, and then washed twice with PBS. These fixed cells were then incubated in blocking solution containing 10% goat serum (Sigma) and 0.1% Triton X-100 (Sigma) for 1 h at room temperature. The cells were then left overnight at 4 °C in blocking solution containing the primary antibody. The next day, the cells were washed three times with PBS and then incubated with the secondary antibody for 1 h at room temperature. The primary antibodies used are given in Supplementary Table S1. Goat anti-mouse IgG, goat anti-rabbit IgG, (1:1000, Life Technologies) conjugated to Alexa Fluor-488 or Alexa Fluor-594 were used as secondary antibodies. Nuclear staining was performed with DAPI (Life Technologies).

Alkaline phosphatase staining

Alkaline phosphatase is an enzyme expressed by ESCs and is used as a marker of pluripotency. To evaluate the alkaline phosphatase expression, the cells were fixed in 10% Neutral Formalin Buffer for 15 min at 4°C, and washed three times with distilled water. These fixed cells were then incubated for 45 min at room temperature in 2ml of the staining solution prepared as follows: 0,005g Naphthol AS MX-PO4 (Sigma, N5000), 0,03g Red Violet LB salt (Sigma, F1625), 200 μl N,N-Dimethylformamide (DMF, Fischer Scientific, D1191), 25 ml of Tris-HCl (MW=157.6, pH 8.3, 0.2M), and 25 ml of distilled water. The alkaline-phosphatase-positive cells showed a red colour and were visible under phase-contrast microscopy.

RNA extraction and quantitative PCR detection of mRNA

RNA was extracted and purified using RNAeasy kits (QIAGEN), according to the manufacturer instructions. Total RNA was treated with DNAse I (Qiagen) to prevent DNA contamination.

The cDNA was produced with SuperScript II Reverse Transcriptase kits (Life Technologies) starting from 1 μg mRNA. Real-time quantitative PCR reactions from 8,3 ng of cDNA were set up in triplicate using a LightCycler DNA SYBR Green I Master PCR machine (Roche). For oligos sequences see Table S2.

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Cell lysis and immunoblotting

Cells were harvested and washed twice with PBS. Cell lysis was performed on ice for 25 min, in RIPA buffer (150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM Tris-HCl, pH 8.0) containing a protease inhibitory cocktail (Roche). Insoluble material was pelleted by centrifugation at 16,000× *g* for 3 min at 4 °C. Protein concentrations were determined using the Bradford assay (Bio-Rad). Thirty micrograms extract was mixed with 4× sample buffer (40% glycerol, 240 mM Tris/HCl, pH 6.8, 8% SDS, 0.04% bromophenol blue, 5% β-mercaptoethanol), denatured at 96ºC for 5 minutes, separated by SDS-PAGE, and transferred to nitrocellulose membranes (PROTRAN-Whatman, Schleicher&Schuell). The membranes were blocked with 5% non-fat dry milk in TBS-T for 60 min, incubated with primary antibodies overnight at 4 °C, washed three times with TBS-T for 10 min, incubated with the peroxidase-conjugated secondary antibody (1:2000; Amersham Biosciences) in TBST with 5% non-fat dry milk for 60 min, and washed three times with TBST for 10 min. Immunoreactive proteins were detected using Supersignal West Dura HRP Detection kits (Pierce).

Chromatin immunoprecipitation assay

ChIP was carried out as described in (Morey et al, 2012). Briefly, ESCs were trypsinised and crosslinked in 1% formaldehyde for 10 min at room temperature. Crosslinking was quenched with 0.125 M glycine for 5 min. The pelleted cells were lysed in 1 ml ChIP buffer and sonicated for 10 min in a Bioruptor sonicator (Diagenode). The soluble material was quantified using Bradford assays. To immunoprecipitate the transcription factors, 500 μg protein was used. Antibodies

were incubated overnight with the chromatin. The immunocomplexes were recovered with 30 µl protein A or G agarose bead slurries. The immunoprecipitated material was washed three times with low-salt buffer and one time with high-salt buffer. DNA complexes were decrosslinked at 65 °C for 3 h, and the DNA was then eluted in 200 µl water using the PCR purification kit (QIAGEN). Two microliters DNA was used for each qPCR reaction, using SYBR green (Fermentas). For oligos sequences see Table S2.

Statistical Analysis.

Averages from three independent experiments were calculated for most of the shown experiments and Student's t-tests were performed for statistical analysis. $p < 0.05$ defined statistical significance.

Supplementary Table 1. Antibodies used in the article with corresponding working dilution.

Antibodies	Working dilution/ Concentration	Company (Catalog number)
Mouse monoclonal Antibody (Ab)		
microtubule-associated protein 2 (MAP2)	1:200 (IF)	Abcam (ab11267)
CD90.2 (Thy-1.2)	0,06 µg x 10^6cells (IF)	eBioscience (12-0902)
β-catenin	1:500 (WB)	BD (MAB-318)
stage-specific embryonic antigen 1 (SSEA-1)	1:100 (IF)	Santa Cruz Biotechnology (sc- 21701)
octamer-binding transcription factor-4 (OCT4)	1:200 (IF)	Santa Cruz Biotechnology (sc-5279)
Nestin	1:200 (IF)	Abcam (ab6142)
Albumin	1:500 (IF)	Abcam (ab19196-2)
Tubulin	1:2000 (WB)	Sigma (T0198)
Actin	1:2000 (WB)	Abcam (ab8226)
Antibodies	Working dilution	Company (Catalog number)
Rabbit polyclonal Antibody (Ab)		
GATA-4	1:200 (IF)	Abcam (ab61170)
SRY-box 2 (SOX2)	1:500 (IF)	Abcam (ab97959)
NANOG	1:300 (IF)	Calbiochem (#SC1000)
GFP	1:500 (IF)	Santa Cruz Biotechnology (sc-8332)
TCF1 FL (C6D9)	1:1000 (WB)	Cell Signaling (#2203)
TCF1 Total (C46C7)	1:1000 (WB) 5 µg (ChIP)	Cell Signaling (#2206)
Active β-catenin	1:500 (WB)	Millipore (#05-665)