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Supplemental Information

Endogenous WNT Signals Mediate BMP-Induced and Spontaneous Differentiation of Epiblast Stem Cells and Human Embryonic Stem Cells

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Supplemental Figure Legends

Figure S1, related to Figure 1. (A-D) T-GFP ESCs were differentiated into EpiSCs by culture in FGF2 and ACTIVIN for 4 passages. The cells acquired the characteristic flattened EpiSC morphology (A), lost expression of the ESC markers *Stella* and *Rex1*, and gained expression of the EpiSC markers *Fgf5* and *Dnmt3b* (B). To verify that the cells had truly differentiated, we transferred them back into ESC conditions as a single cell suspension. On average 8 per 100,000 cells regenerated an ESC colony (n=3) (C), in line with the reversal efficiency of epiblast-derived EpiSCs (Bernemann et al., 2011; Greber et al., 2010), indicating complete differentiation. Teratoma assays demonstrated that the cells retained their pluripotency as they produced teratomas containing derivatives of all 3 germ layers (D). E) Immunofluorescence images of 129S2C1a EpiSCs cultured for 3 days with the indicated factors and immunostained as indicated (blue: DAPI). F) Flow cytometry plots of T-GFP EpiSCs treated with WNT3A and IWP2 and analyzed for T-GFP and SSEA1. G) Plot showing the expansion of SSEA1-positive 129S2C1a EpiSCs in the indicated conditions (mean +/- s.e.m.; n=3 independent experiments). Scale bar: 200 µm (A), 100 µm (D,E).

Figure S1

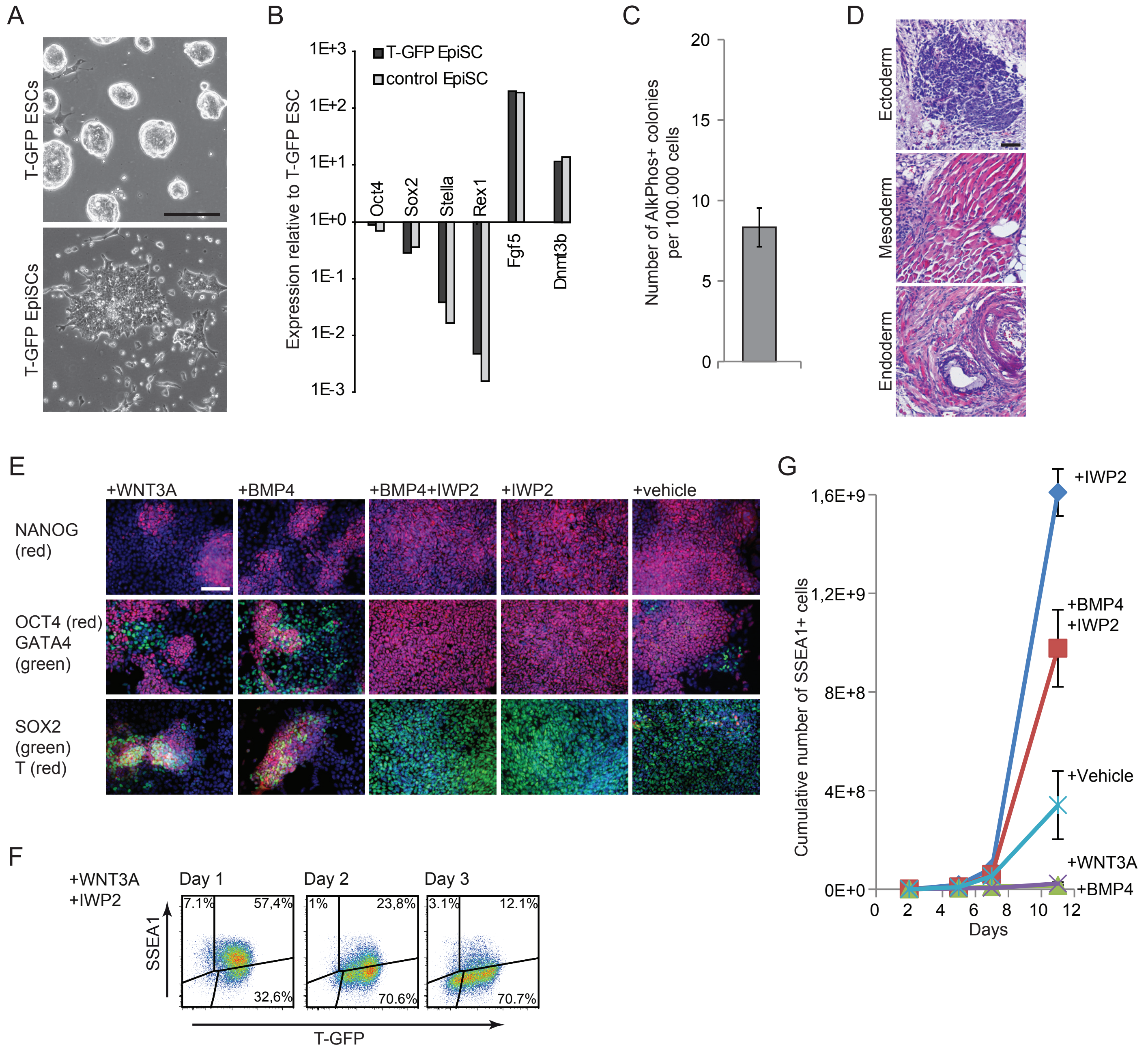


Figure S2, related to Figure 2. A) Expression level of *Wnt* genes in 129S2C1a EpiSCs relative to *Gapdh* (mean +/- s.e.m., n=3; ND, not detected). B) Representative examples of EpiSC colonies obtained from sorted T-GFP EpiSCs, stained for NANOG (red), and quantified in Figure 2A. All colonies stained positive for NANOG, while NANOG-negative cells only formed dispersed clusters. C) Gating strategy relating to Figure 2A.D) Gating strategy relating to Figure 2B. E) Flow cytometry histograms showing several EpiSC lines treated for 3 days with the indicated factors and analyzed for SSEA1. F) Heat map of selected gene expression levels of GFP9 EpiSCs cultured in the presence or absence of IWP2 and analyzed by microarray. Scale bar: 100 µm.

Figure S2

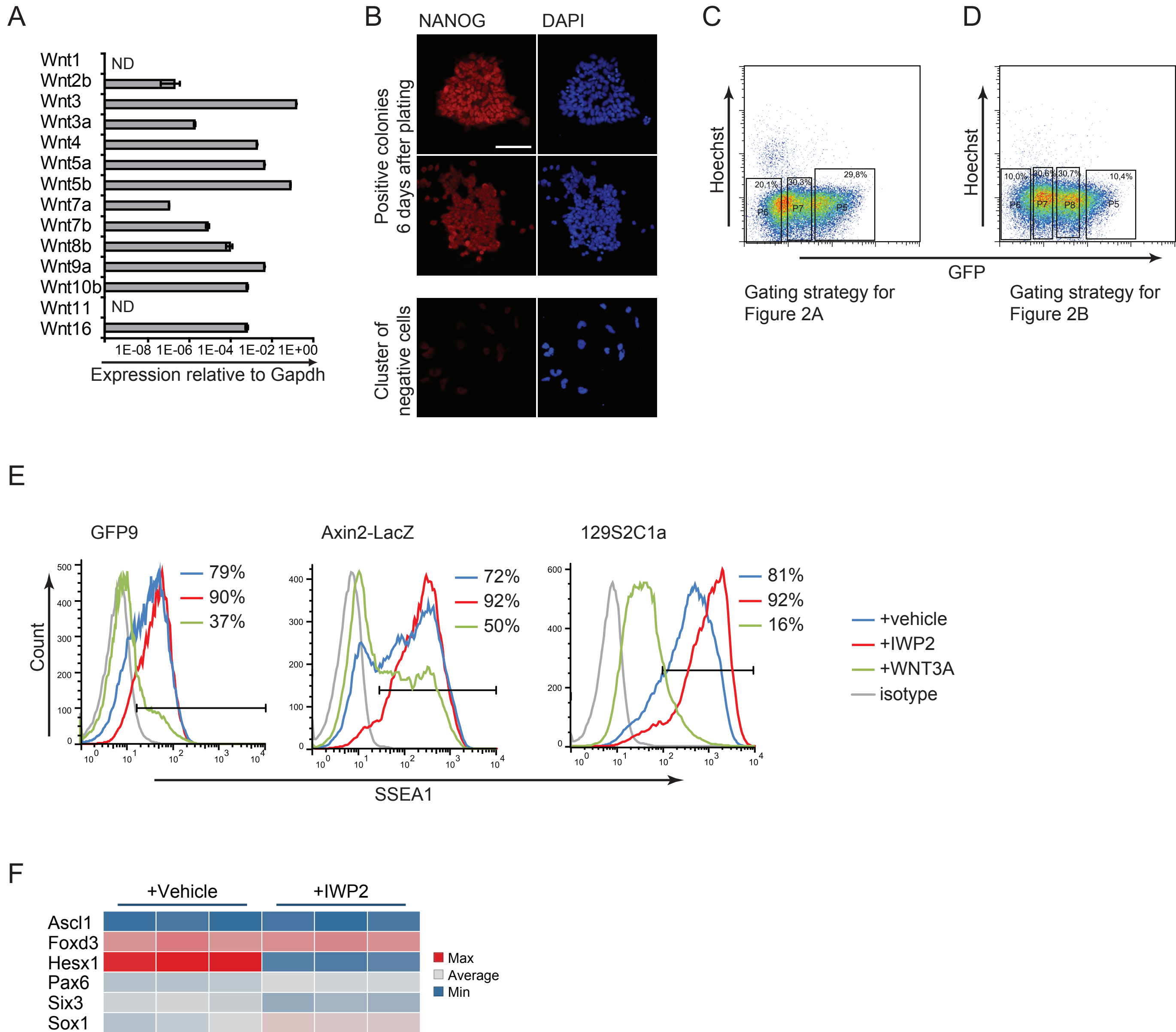
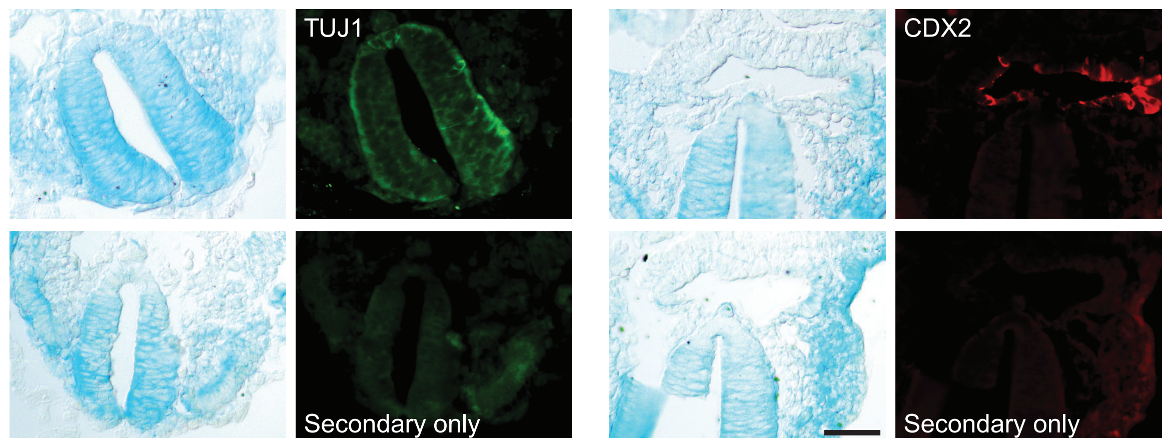
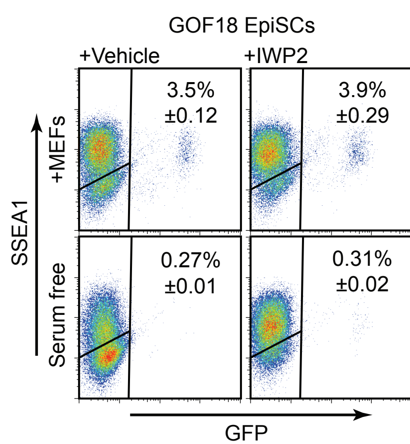


Figure S3, related to Figure 3. A) Immunofluorescence images of sections of X-gal stained chimeras immunostained as indicated. B) Flow cytometry plots of GOF18 EpiSCs cultured at indicated conditions for 4 passages and analyzed for SSEA1 and GFP (mean +/- s.e.m.; n=3 independent experiments). C) Flow cytometry histograms showing percentage of GFP positive cells from GFP-positive and GFP-negative GOF18 EpiSCs analyzed 4 and 18 days after sorting. Scale bar: 50 µm.

Figure S3





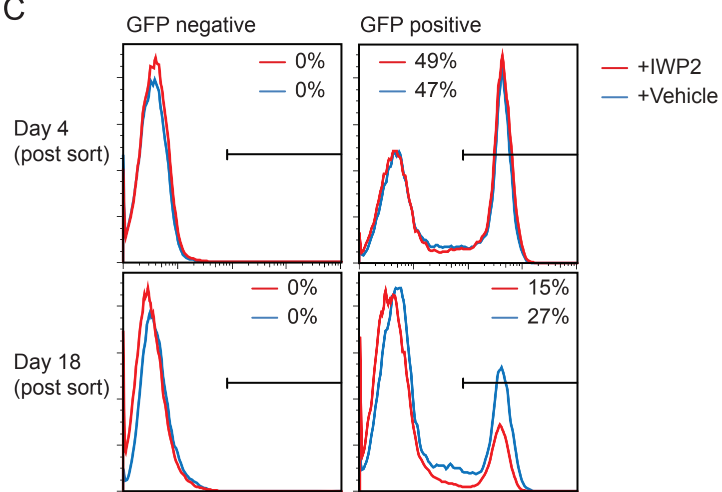


Figure S4, related to Figure 4. A) Representative phase contrast images of H9 (on MEFs) or H1 (mTESR1) hESCs cultured for 6 days at the indicated conditions. Arrows indicate differentiating areas of the colonies. B,C) Immunofluorescence images of H1 (B) or H9 (C) hESCs cultured for 6 days with the indicated factors and immunostained as indicated (blue: DAPI). D) Flow cytometry histograms showing H9 hESCs cultured in the indicated conditions for 7 days and analyzed for NANOG, OCT4 and SOX2. E) Real-time RT-PCR gene expression profile of H9 hESCs cultured for 6 days in the presence of IWP2, plotted relative to cells maintained in the absence of IWP2 (+/- s.e.m.; n=3). F) Percentage of H9 hESCs triple positive for NANOG, OCT4 and SOX2, cultured using the indicated conditions and procedures (mean +/- s.e.m.; n=3 independent experiments). G) Percentage of H9 hESCs expressing SSEA1 cultured using the indicated conditions and procedures (mean +/- s.e.m.; n=3 independent experiments). H) Flow cytometry histograms for SSEA1 and pluripotency transcription factors in H9 and H1 hESCs cultured using the indicated conditions and procedures. Pluripotency factor expression was determined either at day 19 (H9) or day 21 (H1). I) H9 or H1 hESCs were cultured in the presence of IWP2 for 10 passages and then used to establish teratomas. Hematoxylin and eosin staining shows the presence of all 3 germ layers in the teratomas. Scale bar: 200 μm (A,C and I), 100 μm (B).

Figure S4

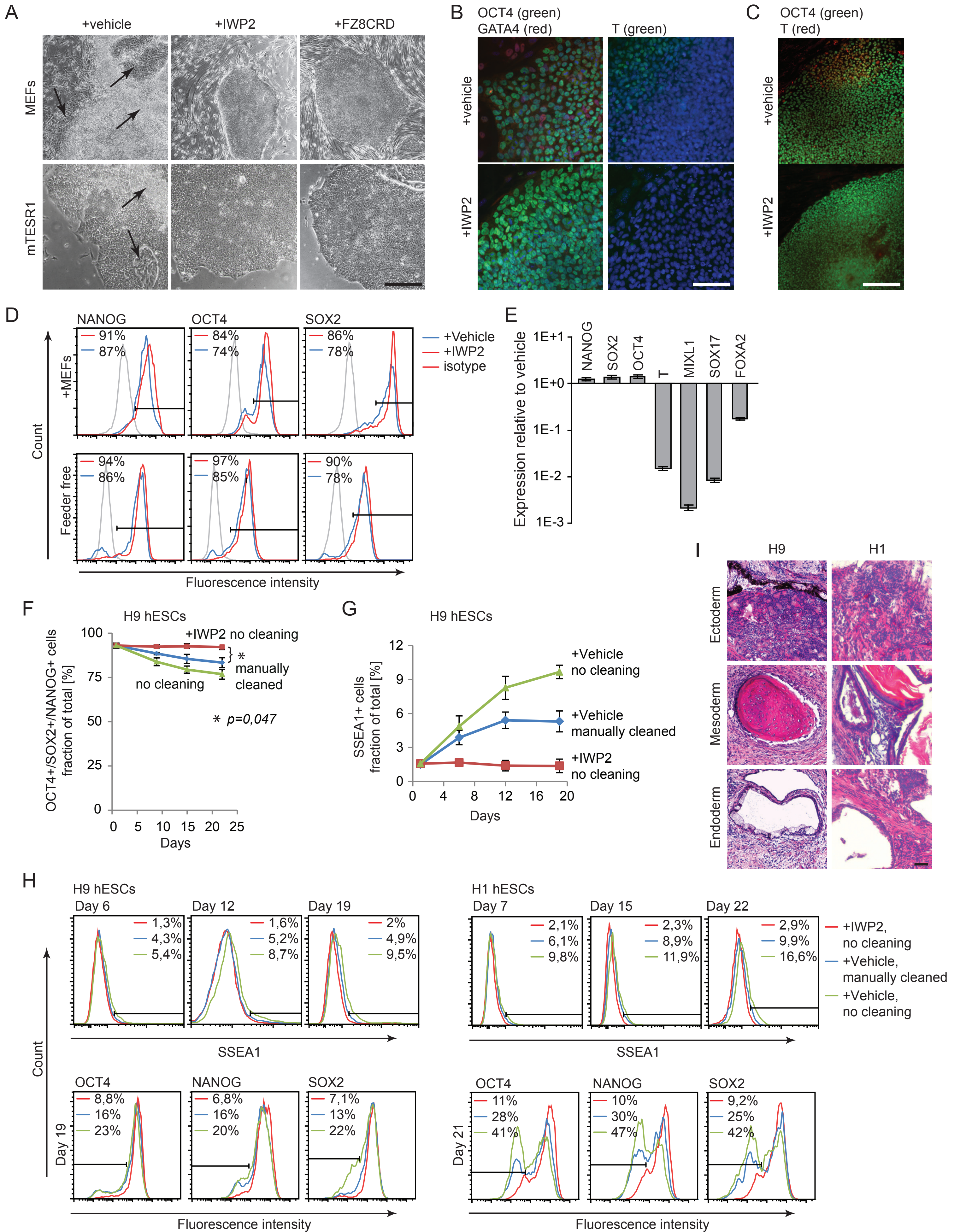
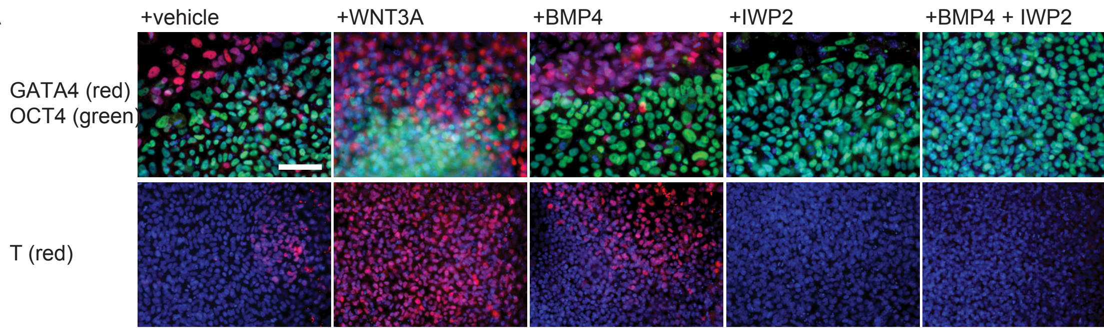
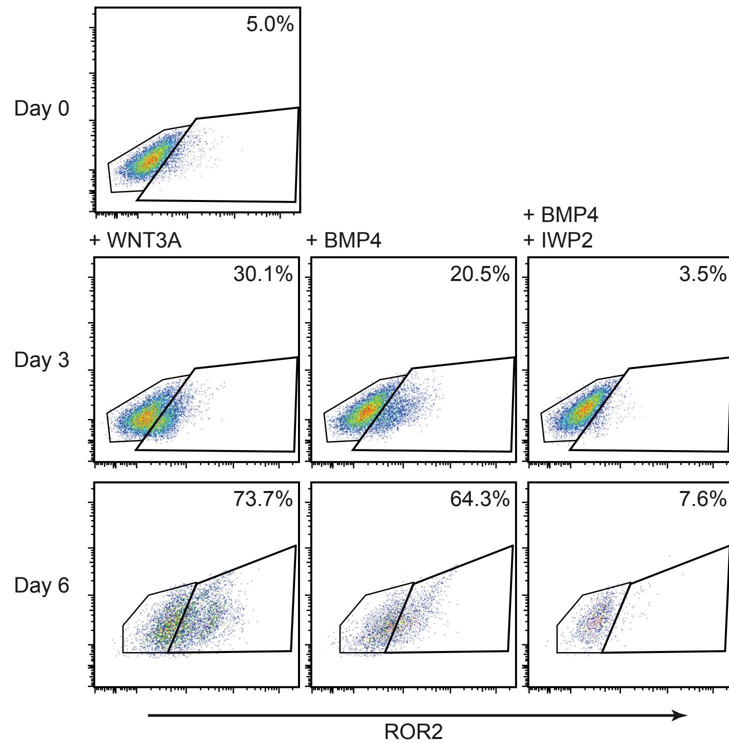
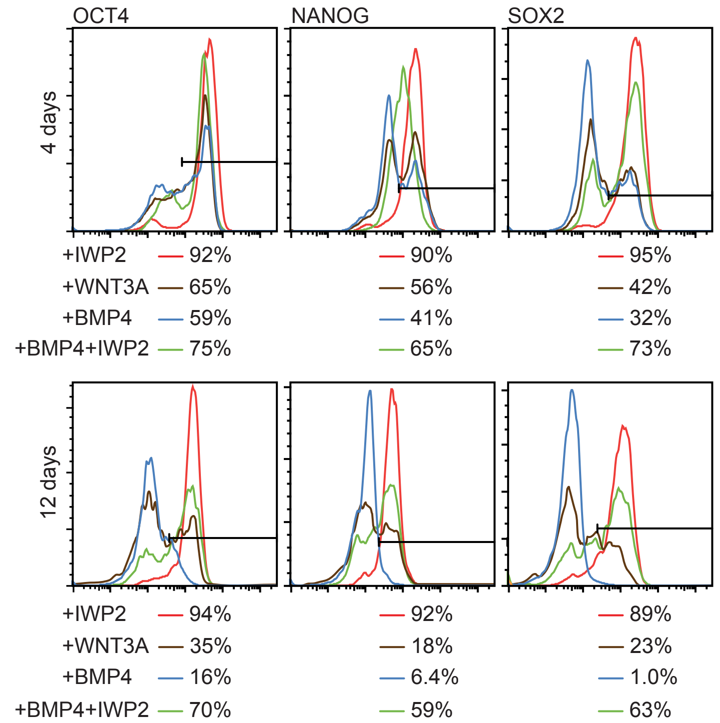


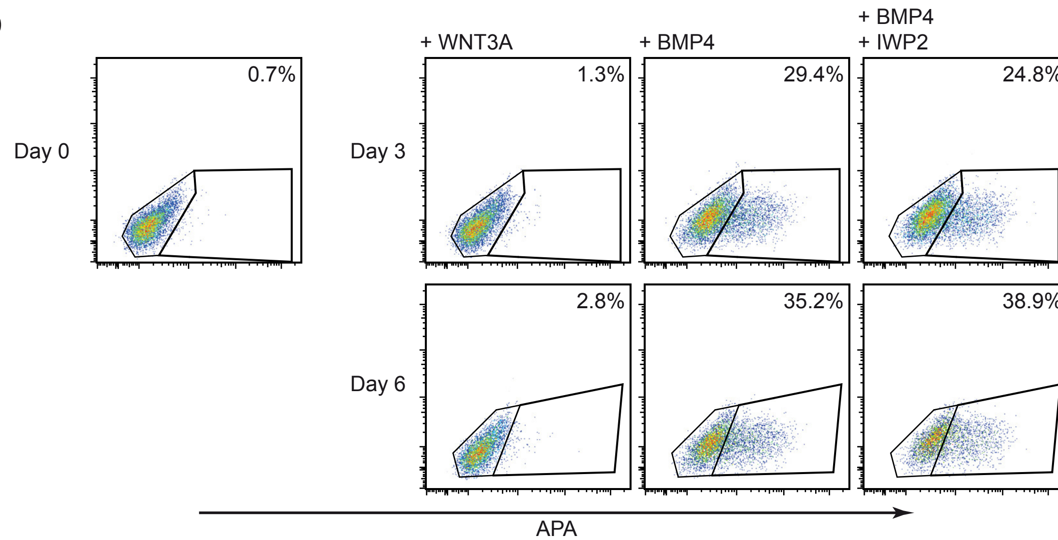
Figure S5, related to Figure 5. A) Immunofluorescence images of H1 hESCs cultured with the indicated factors for 7 days and immunostained as indicated (blue: DAPI). B) Flow cytometry plots from the experiment shown in Figure 5A, showing H1 hESCs cultured with the indicated factors for 3 or 6 days and analyzed for ROR2. C) Flow cytometry histograms showing H1 hESCs cultured with the indicated factors for 4 and 12 days and analyzed for NANOG, OCT4 and SOX2. D) Flow cytometry plots from the experiment shown in Figure 5E, showing H1 hESCs cultured with the indicated factors for 3 or 6 days and analyzed for APA. E) Flow cytometry histograms showing H1 hESCs differentiated with BMP4 in the presence of IWP2 for 5 and 7 days and analyzed for APA (mean +/- s.e.m.; n=3 independent experiments). Scale bar: 100 µm.

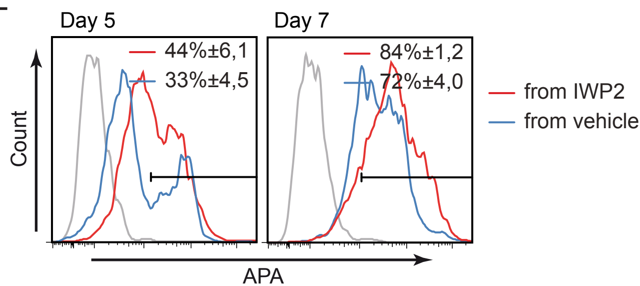
Figure S5











Supplemental Table Legends

Table S1. Genes Differentially Expressed between EpiSCs Maintained in the Presence or Absence of IWP2, Related to Figure 2.
This table uses a threshold value of 0.05 for false discovery rate-adjusted p value and minimum fold change of 2. The list contains 515 probe sets representing 408 unique gene symbols.

Table S2. Gene Set Enrichment Analysis for Gastrulation Markers in EpiSCs Maintained in the Absence versus the Presence of IWP2, Related to Figure 2.

Table S3. Gene Set Enrichment Analysis for Definitive Endoderm Markers in EpiSCs Maintained in the Absence versus the Presence of IWP2, Related to Figure 2.

Table S4. Gene Set Enrichment Analysis for Markers Expressed in Endoderm and Absent from Mesoderm and Ectoderm in EpiSCs Maintained in the Absence versus the Presence of IWP2, Related to Figure 2.

Table S5. Gene Set Enrichment Analysis for Markers Expressed in Mesoderm and Ectoderm and Absent from Endoderm in EpiSCs Maintained in the Absence versus the Presence of IWP2, Related to Figure 2.

Supplemental Experimental Procedures

Cell culture. Epiblast stem cells (EpiSCs) were cultured on gelatin and fetal calf serum coated plates in EpiSC medium: N2B27 medium supplemented with 20 ng/ml ActivinA and 12 ng/ml bFGF (both Peprotech). N2B27 medium consisted of one volume DMEM/F12 combined with one volume Neurobasal medium, supplemented with 0.5% N2 Supplement, 1% B27 Supplement, 0.033% BSA 7.5% solution, 50 μM β-mercaptoethanol, 2 mM Glutamax, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Invitrogen). EpiSCs were passaged 1:4–1:10 every 3 days by triturating the colonies into small clumps using 0.5 mg/ml collagenase IV (Sigma).
To differentiate EpiSCs from ES cells, trypsinized ES cells were seeded at a density of 10,000 cells/cm^2 on gelatin and FCS coated plates in EpiSC medium supplemented with 1,000 U/ml LIF for the first passage. The cells were then passaged 1:4–1:10 every 3 days as small clumps using 0.5 mg/ml collagenase IV.
To revert EpiSCs to ESCs, EpiSCs were triturated to a single cell suspension in 0.5 mg/ml collagenase IV (Sigma) and seeded at a density of 2,000 cells/cm^2 in EpiSC medium on MEFs supplemented with 10μM the ROCK inhibitor Y-27632 (Stemgent). The next day, the medium was changed to N2B27 medium supplemented with LIF (1,000 U/ml), PD0325901 and CHIR99021. After 4 days the cells were stained for alkaline phosphatase activity using the SCR004 kit (Millipore) and the number of alkaline phosphatase-positive colonies counted.
Mouse ESCs were cultured in N2B27 medium supplemented with 1,000 U/ml LIF (Chemicon) and 250 ng/ml Wnt3a protein on gelatin coated plates.
WA01 (H1) and WA09 (H9) hESCs were cultured on irradiated mouse embryonic fibroblast feeder layers in medium consisting of DMEM/F12 supplemented with 20% Knockout Serum Replacement, 2 mM Glutamax, 1x MEM non-essential amino acids, 50 μM β-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Invitrogen) and supplemented with 10 ng/ml human bFGF (Millipore). For differentiation experiments cells were cultured on a layer of Matrigel (BD Biosciences) in medium consisting of DMEM/F12 supplemented with 20% Serum (HyClone), 2 mM Glutamax, 1x MEM non-essential amino acids, 50 μM β-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Invitrogen) and supplemented with 250 ng/ml Wnt3a, 50 ng/ml BMP4, or 2 μm IWP2, as indicated.
For feeder free culture, hESCs were cultured on a layer of Matrigel (BD Biosciences) in mTeSR1 medium (Stemcells Technologies) or TeSR-E8 medium (Stemcells Technologies).
Media, recombinant proteins and small molecules were changed daily in all experiments except when indicated otherwise. IWP2 (Merck or Stemgent), CHIR99021 (Axon Medchem or Stemgent), PD0325901 (Merck), LDN-193189 (Stemgent) and ROCK inhibitor Y-27632 (Stemgent) were diluted from 2 mM (IWP2), 0.2 mM (LDN-193189) or 10 mM (others) stocks in water (Y-27632) or dimethylsulphoxide (all others), and used at 2, 3, 0.9, 0.2 and 10 μM, respectively. Bmp4 (Invitrogen) was used at 15 ng/ml for EpiSCs, and 50 ng/ml for hESCs. Wnt3a and Fz8CRD proteins were used at 250 and 1,000 ng/ml, respectively. Recombinant mouse Wnt3a protein was produced in Drosophila S2 cells grown in suspension culture, and purified by Blue Sepharose affinity and gel filtration chromatography as described (Willert et al., 2003). Fz8CRD was produced as an Fc fusion protein as described (Hsieh et al., 1999).
Epiblast Stem Cell derivation. Epiblasts were dissected from E6.5 Rosa26-LacZ (Soriano, 1999) or Actin-GFP (Okabe et al., 1997) embryos and collected in DMEM with 10% FCS and 20 mM HEPES. After dissociation into small clumps using a brief trypsin-EDTA treatment, the clumps were plated on a MEF feeder layer in DMEM supplemented with 18% Knockout Serum

Replacement, 2% fetal bovine serum (Hyclone), 1 mM sodium pyruvate, 1x MEM non-essential amino acids, 50 µM β-mercaptoethanol, 100 U ml/1 penicillin, 100 µg ml/1 streptomycin (all from Invitrogen), 12 ng/ml bFGF (Peprotech) and 2 µM IWP2 (Stemgent). Four to seven days after initial plating, the epiblast outgrowths were passaged using 0.5 mg/ml collagenase IV and expand in MEF-containing culture. The MEFs were plated in the presence of 2 µM IWP2 prior to start of derivation or passaging to prevent the accumulation of endogenous Wnt proteins. Usually EpiSCs were transferred to feeder-free N2B27 media after passage 2.

EpiSCs and hESCs self-renewal assays. To quantify self-renewal over multiple passages, EpiSCs or hESCs were initially plated in 12-well plates at 1:10 ratio and the total number of cells was estimated. Every 2-3 days for EpiSCs and 5-7 days for hESCs cells were passaged at ratio that would result to a similar density (between 1:4 to 1:10 ratio) and at the same time cells were stained with SSEA1 or nuclear pluripotency markers for FACS analysis (see FACS marker staining). The cumulative number of positive cells was calculated by multiplying the cell counts by the dilution factor used for passaging.

Teratoma formation. EpiSCs and hESCs were harvested using 0.5 mg/ml collagenase IV (Sigma) and resuspended in PBS or 30% BD Matrigel in PBS (BD Biosciences), respectively. Between $2x10^6$ and $6x10^6$ cells were subcutaneously injected into the flank of NOD/SCID mice. After 5-8 weeks teratomas were removed, fixed in 4% PFA, embedded in paraffin, sectioned and stained with hematoxylin and eosin. All animal procedures were performed in accordance with institutional and national guidelines and regulations, and approved by the Erasmus MC Animal Experiment Committee (DEC).

Blastocyst injection. EpiSCs were recovered by treatment with Enzyme-Free Cell Dissociation Buffer (Gibco), and 10-15 cells were injected into blastocysts collected from C57Bl/6 mice. Blastocysts (10-15 per mouse) were transferred into the uterus of a pseudopregnant mouse and embryos recovered 7 days later.

Embryoid body formation. T-GFP and 7xtcf-GFP ESCs were triturated to a single cell suspension using trypsin-EDTA and resuspended in differentiation medium composed of DMEM plus 15% fetal bovine serum (Hyclone), 1x MEM non-essential amino acids, 50 µM β-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin (all from Invitrogen). Hanging drops were prepared from 2000 ESCs per 20 µl drop in differentiation medium. After 3 days embryoid bodies were transferred to low attachment plates for further culture in a shaking incubator. The embryoid bodies were collected at indicated time points and dissociated into single cells using collagenase IV in 5% FCS/PBS (5 minutes in 37°C) and collected for flow cytometry. Following markers were used for staining: SSEA1-PE (eBioscience, 1:50) and CD31-PE-Cy7 (eBioscience, 1:2000) in 5% FCS/PBS for 40 minutes at 4°C. 1µg/ml Hoechst 33258 (Molecular Probes) was used for live/dead cells assessment. Cells were analyzed using a FACSAria III flow cytometer and results analyzed by FlowJo.

Correlation between level of Brachyury reporter and ability to form EpiSC colonies or embryoid bodies. T-GFP EpiSCs were plated in N2B27 medium supplemented ActivinA and bFGF for 3 days, dissociated with 0.5 mg/ml collagenase IV (Sigma) for EpiSC colony formation assay or by 0.25% Trypsin-EDTA for embryoid body formation assay, resuspended in PBS with 10% serum and 1 µg/ml Hoechst 33258, and live cells sorted into 3 or 4 categories based on GFP intensity using a FACSAria III cell sorter (BD Biosciences). The gating strategies are shown in Figures S2D and S2E. Sorted cells were seeded at a density of 3000 or 6000 cells/cm^2 in N2B27 medium supplemented with bFGF, Activin A, IWP2 and ROCK inhibitor. After 6 days the cells were immunostained for Nanog and the number of positive colonies was counted by eye. For

embryoid body formation, 6000 sorted cells per 25 µl droplet were aggregated in hanging drops for 3 days, and the embryoid bodies collected and photographed.

Flow cytometry. For live flow cytometry experiments of EpiSCs, single cell suspensions were made using trypsin-EDTA (5 minutes in 37°C), washed with 5% FCS/PBS, and stained with SSEA1-PE (eBioscience, 1:50) for 40 minutes at 4°C. 1µg/ml Hoechst 33258 or 7AAD was used for live/dead cells assessment. Prior to the time course experiment of T-GFP EpiSCs treated with gastrulation-inducing factors, cells were cultured in the presence of IWP2 for one passage.

For live flow cytometry experiments of hESCs, single cell suspensions were made using Enzyme-Free Cell Dissociation Buffer (Gibco) for 30 min at 37ºC, washed with 5% FCS/PBS and stained with anti-APA or anti-ROR2 as described previously (Drukker et al., 2012). For the nuclear pluripotency markers, EpiSCs and hESCs were stained using Mouse or Human Pluripotent Stem Cell Transcription Factor Analysis Kits (BD Biosciences) according to the manufacturer's protocol. Cells were analyzed using FACSAria III, FACS Fortessa, or FACS SCAN flow cytometers (BD Biosciences), and data analyzed using FlowJo.

Real time RT-PCR analysis. Total RNA was prepared using a QIAGEN RNeasy mini kit with on-column DNase digestion, or using TriPure (Roche) according to the manufactures protocol, followed by reverse transcription using Superscript II (Invitrogen). Quantitative PCR was carried out on a Roche Lightcycler 480 using Lightcycler 480 SYBR Green Master mix (Roche). Relative quantification was carried out using Gapdh as a reference gene. All PCRs were carried out in triplicate, and the mean crossing point was used for quantification. Primer sequences were designed such that they spanned splice junctions whenever possible and are provided in Table S3.

Immunohistochemistry. Cells were fixed with 4% paraformaldehyde for 10 min at 4°C, permeabilized for 10 min with ice-cold methanol, washed with PBS/0.5% Triton X-100 (PBT), and blocked with 1% BSA (Sigma) and 5% normal donkey serum in PBT (blocking solution) for 1 hour. Samples were then incubated with primary antibody in blocking solution overnight at 4°C, washed three times with PBT, and primary antibodies detected by DyLight-488 or -594 labelled secondary antibodies (Jackson ImmunoResearch), followed by imaging. Antibodies and concentrations: goat-anti-Oct4 (Santa Cruz sc-8628, 1:250), rabbit-anti-Nanog (REC-RCAB0002P-F Cosmo Bio, 1:250), rabbit-anti-T (Santa Cruz sc-20109, 1:100), goat-anti-T (Santa Cruz sc-17743, 1:100) and rabbit-anti-Gata4 (Santa Cruz sc-9053, 1;100), goat-anti-Sox2 (Immune Systems, 1:1000), rabbit-anti- E-cadherin (Cell Signaling 24E10, 1:200), rabbit-anti-Neuronal Class III B-tubulin (Covance PRB-435P, 1:500), mouse-anti-Cdx2 (Biogenex, MU392A-UC 1:400). Human ESCs were stained for alkaline phosphatase according to manufacturer's protocol (Millipore, Alkaline Phosphatase Detection Kit SCR004).

X-gal staining. Embryos were fixed for 30 min and cells for 1 min in 0.5% glutaraldehyde and 1% paraformaldehyde in PBS, washed in PBS and incubated in X-Gal solution composed of 0.1% 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside, 0,02% Nonidet™ P 40, 0.01% Sodium Deoxycholate, 2mM magnesium chloride, 5mM potassium hexacyanoferrate(III), 5mM potassium hexacyanoferrate(II) trihydrate, 1mM ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (all from Sigma) for 5 h to overnight at room temperature. Stained cells were counterstained with neutral red and photographed with an Olympus BX40 light microscope.

Micro-array analysis. Total RNA was prepared using TriPure (Roche) according to the manufacturer's protocol from GFP9 EpiSCs maintained in the presence or absence of IWP2 for 2 passages in EpiSC medium. RNA was converted to biotin-labeled cRNA, hybridized on the Affymetrix Mouse Genome 430 2.0 Array, and analyzed with the Affymetrix GeneChip Scanner 3000 at the Erasmus MC Center for Biomics (Rotterdam, The Netherlands) according to manufacturer's instructions. Raw intensity values were normalized by calling Variance

Stabilization (VSN) and calculating the summaries with the medianpolish algorithm of the Robust Multi-Array Average (RMA) expression (Huber et al., 2002; Irizarry et al., 2003). The probe sets gene annotations were based on Ensembl release 75 (Flicek et al., 2014). Quality control was investigated using the *qc* function from the *simpleaffy* R package. Differentially expressed genes were called with the *limma* package, using a threshold value of 0.05 for False Discovery Rate (FDR) adjusted p-value and minimal fold change of 2.
Gene Set Enrichment Analysis (Subramanian et al., 2005) was run on the vehicle versus IWP treated EpiSCs, with 1000 permutations per run. Permutation type was set to "phenotype". The enrichment statistic used was weighted and signal to noise was used as our preferred metric for gene ranking.
To combine gene expression data from Affymetrix GeneChip and Illumina BeadArray platforms, we used a similar approach as described previously (Heider and Alt, 2013). We first downloaded 112 gene expression datasets deposited in the Gene Expression Omnibus (GEO) database (GSE46227) (Kojima et al., 2014) and combined these data with our 9 Affymetrix GeneChip datasets. The probes and probesets were combined using the probe(set)s/gene annotation of Ensembl release 75 (Flicek et al., 2014). In each sample, genes with multiple probe/probesets were collapsed to their median value. This resulted in 17058 distinct genes from both platforms. Second, we quantile normalized the combined expression data (Bolstad et al., 2003). Third, we applied the Empirical Bayes method (Johnson et al., 2007) in a unsupervised mode using the CrOss-platform NOrmalization in R (CONVOR) package (Rudy and Valafar, 2011). We verified the proper unsupervised hierarchical clustering with euclidean distance of the cross-platform normalized data (data not shown). Subsequently we selected the gene expression values from EpiSCs and Epiblast/Ectoderm samples cultured in Tam's and our lab to conduct a Principal Component Analysis (PCA). Using R, the PCA was calculated based on the covariance matrix of 1117 genes that are differentially expressed with a FDR <0.05 and a fold change of at least 1.5.
RNA Seq. Total RNA was prepared using TriPure (Roche) according to the manufacturer's protocol from GFP9 EpiSCs treated for 48 hrs with Bmp4, Wnt3a, and/or IWP2 in EpiSC medium. RNA-Seq was performed at the Erasmus MC Center for Biomics (Rotterdam, The Netherlands) according to manufacturer's instructions (Illumina). Briefly, polyA containing mRNA molecules were purified using poly-T oligo attached magnetic beads. Following purification, the mRNA was fragmented into ~200 bp fragments using divalent cations under elevated temperature. The cleaved RNA fragments were copied into first strand cDNA using reverse transcriptase and random primers. This was followed by second strand synthesis using DNA polymerase I and RNaseH treatment. These cDNA fragments were end repaired, a single A base was added and Illumina adaptors were ligated. The products were purified and size selected on gel and enriched by PCR. The PCR products were purified by Qiaquick PCR purification and used for cluster generation according to the Illumina cluster generation protocols (www.illumina.com). The sample was sequenced for 36bp and raw reads were uploaded on the Galaxy main server (www.usegalaxy.org) (Goecks et al., 2010) and a standard pipeline for RNA-Seq analysis was applied, using modules from the Tuxedo suite. Samples were filtered and trimmed with FASTQ. The resulting files were aligned to the mm9 reference genome build using preSet settings. The aligned reads were imported to Cufflinks, using multi-read and effective length correction, and FPKM values calculated. The mm9 build was used as a reference annotation. Principal component analysis on the FPKM values was performed using Tibco Spotfire on the 9939 genes that had a FPKM value of 5 or more in at least one of the 4 samples.

PCR primer sequences		
Mouse	**Forward**	**Reverse**
Gapdh	TATGATGACATCAAGAAGGTGG	CATTGTCATACCAGGAAATGAG
Oct4	GAACATGTGTAAGCTGCGG	CAGACTCCACCTCACACG
Sox2	AGCTCGCAGACCTACATGAA	CCCTGGAGTGGGAGGAA
Nanog	AAAGGATGAAGTGCAAGCG	TCTGGCTGCTCCAAGTT
Fgf5	AATATTTGCTGTGTCTCAGG	TAAATTTGGCACTTGCATGG
Otx2	CATGATGTCTTATCTAAAGCAACCG	GTCGAGCTGTGCCCTAGTA
Dnmt3b	CCAAGGACACCAGGACGCGC	TCCGAGACCTGGTAGCCGGAA
Rex1	GCTCCTGCACACAGAAGAAA	GTCTTAGCTGCTTCCTTCTTGA
Pecam1	CAAAGTGGAATCAAACCGTATCT	CTACAGGTGTGCCCGAG
Stella	TTCAAAGCGCCTTTCCCAA	ACATCTGAATGGCTCACTG
Wnt1	ATGAACCTTCACAACAACGA	GGCGATTTCTCGAAGTAGAC
Wnt2b	CATGAACTTACACAACAACC	CAAAGTAGACAAGATCAGTCC
Wnt3	CAAGCACAACAATGAAGCAG	GGAGTTCTCGTAGTAGACCA
Wnt3a	AGTGAGGACATTGAATTTGG	GTTTCTCTACCACCATCTCC
Wnt4	GAATCTTCACAACAACGAGG	ATCTGTATGTGGCTTGAACTG
Wnt5a	TAATTCTTGGTGGTCTCTAGGT	GCACCTTCTCCAATGTACTG
Wnt5b	TATGCAGATAGGTAGCCGAG	TTGTTCTGTAGGTTCATGAGAG
Wnt7a	CATCATCGTCATAGGAGAAGG	GATAATCGCATAGGTGAAGG
Wnt7b	CATGAACCTTCACAACAATGAG	TTGTACTTCTCCTTGAGCAG
Wnt8b	GTACACCCTGACTAGAAACTG	ATTGTTGTGCAGATTCATGG
Wnt9a	AGTACAGCAGCAAGTTTGTC	GAGCGAGGTCTCATATTTGTG
Wnt10b	CGGGATTTCTTGGATTCCAG	TTGTGGGTATCGATAAAGATGG
Wnt11	GATCCCAAGCCAATAAACTG	AGATACACAAGTTCTGAGTCCT
Wnt16	CTCTTTGGCTATGAGCTGAG	CGTTGTTGTGTAGATTCATGG
T	GAACCTCGGATTCACATCG	GGCATCAAGGAAGGCTTTAG
Sp5	CGGGACCTATGAGCGCA	TTCGGGCGGAGGAGAAT
Sox17	TGTATGAGTTCTTTGGAGACAAAGTAG	ATAGGAAGGCTGAAATTCAGATG
Snail1	CTTGTGTCTGCACGACCTGT	CTTCACATCCGAGTGGGTTT
Klhl4	CAACAACTGCCACTCCAAATTG	TATGGATGCTGCTAAAGGCAC
Zfp42	TCGGGGCTAATCTCACTTTCAT	CCCTCGACAGACTGACCCTAA
Prdm14	AGCACCCAACCGACTTACAG	GTGGCACATCACCAAATGAG
Tbx3	CAGCAGCCCCCACTAACTG	AGATCCGGTTATCCCTGGGAC
Pou3f1	GGCGCATAAACGTCGTCCA	TCGAGGTGGGTGTCAAAGG
Lefty1	CGCGAAACGAACCAACTTGT	CCAACCGCACTGCCCTTAT
Pitx2	GTCCGTGAACTCGACCTTTTT	GCAGCCGTTGAATGTCTCTTC
Lrp2	GGCTGCATACATTGGGTTTTCA	AAAATGGAAACGGGGTGACTT
Slc39a8	GCCTAAGCATCCAGAGGGAGA	CAGGTATGTCCTGCTGATTGC
Prss23	GGCGTCGAAGTCTGCCTTAG	GGTGAGTCCCTACACCGTTC
Klhl4	CAACAACTGCCACTCCAAATTG	TATGGATGCTGCTAAAGGCAC
Spry4	TCTGGTCAATGGGTAAGATGGT	GCAGCGTCCCTGTGAATCC
Thbs1	CGGGGATCAGGTTGGCATT	GGGGAGATAACGGTGTGTTTG
Fn1	GCCCAGTGATTTCAGCAAAGG	ATGTGGACCCCTCCTGATAGT
Kit	GTCGCCAGCTTCAACTATTAACT	GCCACGTCTCAGCCATCTG
Map2k6	TTGGAGTCTAAATCCCGAGGC	ATGTCTCAGTCGAAAGGCAAG
Fgf15	CTGACACAGACTGGGATTGCT	ATGGCGAGAAAGTGGAACGG
Epas1	TGTGTCCGAAGGAAGCTGATG	CTGAGGAAGGAGAAATCCCGT
Rhox5	CCCTGGTGCCACTATCCTT	ACTCGGAAGAACAGCATGATG
Fgf4	GCTGCTCATAGCCACGAAGAA	GGGCATCGGATTCCACCTG
Klf2	CACGTTGTTTAGGTCCTCATCC	CTCAGCGAGCCTATCTTGCC
Alpl	GGCTACATTGGTGTTGAGCTTTT	CCAACTCTTTTGTGCCAGAGA
Tnfrsf19	AGAAAATTCAGCGCAGATGGAA	TTCTGTGGGGGACACGATG
Krt8	ATCGAGATCACCACCTACCG	TGAAGCCAGGGCTAGTGAGT
Krt19	TGACCTGGAGATGCAGATTG	AATCCACCTCCACACTGACC
Nedd9	CCACAGCACTCAAGGGGTAT	ATGGTGGAATGGCATAGACC
Plat	AGTGGTCTTGGGCAGAACAT	CTGCAGTAATGCGATGTCGT

Mnx1	GTTGGAGCTGGAACACCAGT	CTTTTTGCTGCGTTTCCATT
Lgr5	TAACAGGGAACCGAGCCTTA	CACTGTTGCCGTCGTCTTTA
Vil1	CTGGAAACCGAGACCTTGAG	AGTTTCCCAGCTCTGCCTTA
Fgf8	CCGGACCTACCAGCTCTACA	ACTCGGACTCTGCTTCCAAA
Id1	GAGTCTGAAGTCGGGACCAC	GAGTCCATCTGGTCCCTCAG
Cdh2	GGGGATATTGGGGACTTCAT	GAGTTGAGGGAGCTCAAGGA
Plet1	CTTGACATCCCAAAGCCAGT	GGTTGAGGCTGAGGTTGTA
Cdh1	GCCACCAGATGATGATACCC	GGAGCCACATCATTTCGAGT
Nodal	ACCATGCCTACATCCAGAGC	CATGTCCTTGTGGTGTTCCA
Axin2	AGGAGCAGCTCAGCAAAAAG	GCTCAGTCGATCCTCTCCAC
Human	**Forward**	**Reverse**
GAPDH	GGCCTCCAAGGAGTAAGACC	AGGGGTCTACATGGCAACTG
BRY	GCAAAAGCTTTCCTTGATGC	ATGAGGATTTGCAGGTGGAC
HOXB1	TCCCTGGGAACTTTGACAAC	GCTCTGACACCTTCGCTAGG
WNT3	GCTGACTTCGGCGTGTTAGT	CACTTGCATTTGAGGTGCAT
POU5F1	CGAAAGAGAAAGCGAACCAG	ACACTCGGACCACATCCTTC
KRT7	CAGGAACTCATGAGCGTGAA	CTGCCACCAGTGGAATTCAT
ENPEP	AAGAACATGGCCTGGAATTG	AGCTCTCCATCTGCCACAGT
GCM1	CCTCTGAAGCTCATCCCTTG	GCTCTTCTTGCCTCAGCTTC
SOX2	AACCCCAAGATGCACAACTC	CGGGGCCGGTATTTATAATC
NANOG	CAGAAGGCCTCAGCACCTAC	ACTGGATGTTCTGGGTCTGG
T	TCGGAACAATTCTCCAACCT	GGGTACTGACTGGAGCTGGT
MIXL1	AGTCCAGGATCCAGGTATGGT	GGGGCTTCAGACATTTCGT
FOXA2	GAGGGCTACTCCTCCGTGA	CACGTACGACGACATGTTCA
CGA	GGTGCCCCAATACTTCAGTG	CCCCATTACTGTGACCCTGT
SOX17	AGCAGAATCCAGACCTGCAC	TTGTAGTTGGGGTGGTCCTG
SIX3	CGGGAGTGGTACCTACAGGA	GGTGCTGGAGCCTGTTCTT
HESX1	TAGAGGCCGAAGACCAAGAA	ACGCCGATTTTGAAACCA
FOXD3	ACTCTGCCTCTCCCCAATTT	TCGGTTTTCGGTTTTACCTG
SOX1	AAAGTCAAAACGAGGCGAGA	AAGTGCTTGGACCTGCCTTA
PAX6	GCCAGCAACACACCTAGTCA	TGTGAGGGCTGTGTCTGTTC
ASCL1	GGACGAGGGCTCTTACGAC	AACGCCACTGACAAGAAAGC

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