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Supplemental Information

OCT4 Coordinates with WNT Signaling to Pre-pattern Chromatin at the *SOX17* Locus during Human ES Cell Differentiation into Definitive Endoderm

Lei Ying, Jason A. Mills, Deborah L. French, and Paul Gadue

Supplemental Figures

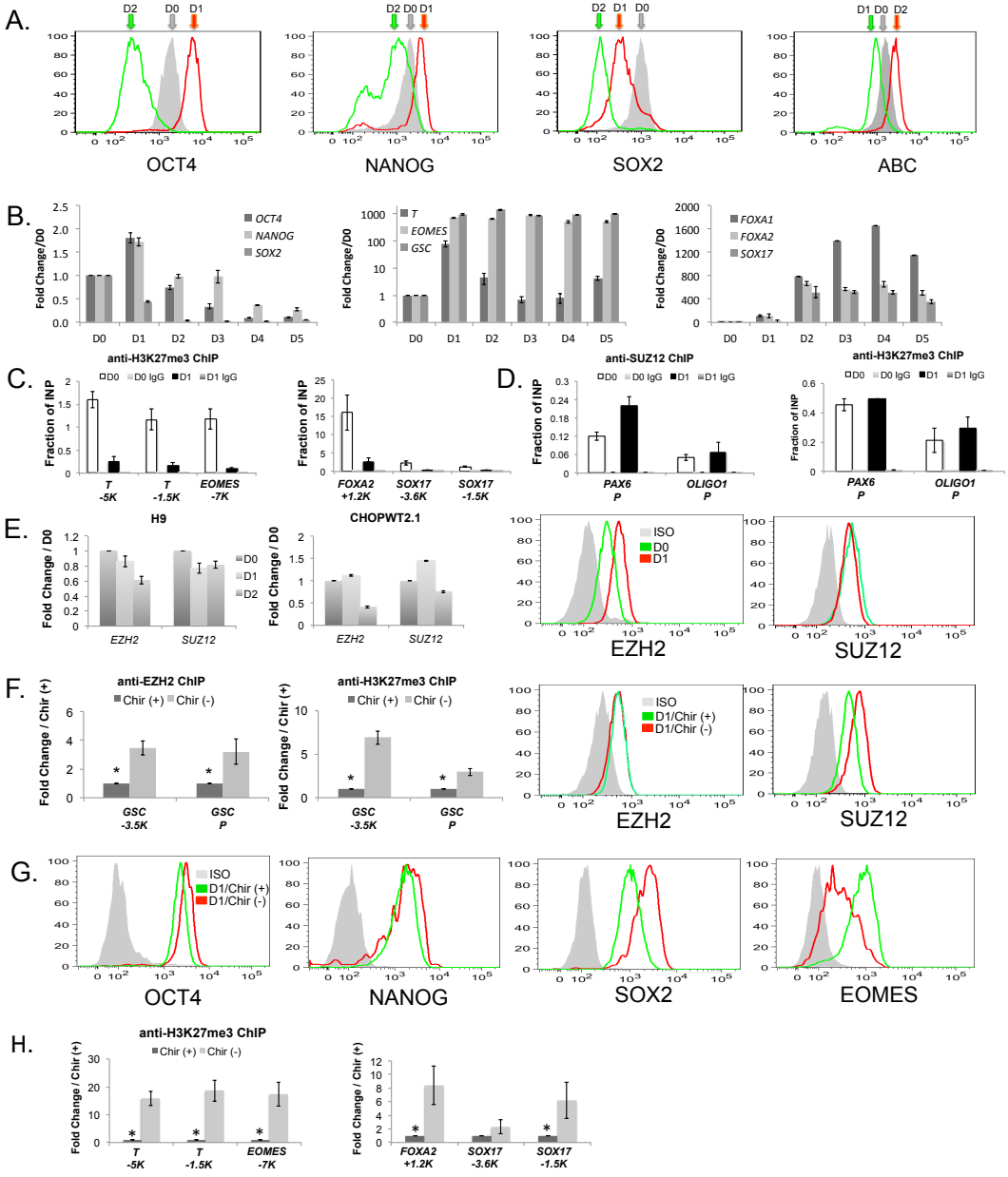

Figure S1: Gene expression profiles and ChIP-qPCR data during endoderm differentiation.

A. Flow cytometry analysis of OCT4, NANOG and SOX2 and activated β-Catenin (ABC) on D0, D1 and D2 H9 endoderm differentiation cultures. B. Time course qRT-PCR expression profiles of pluripotency genes (*OCT4, NANOG* and *SOX2*), primitive streak genes (*T, EOMES,* and *GSC*) and endodermal genes (*FOXA1, FOXA2* and *SOX17*) in H9 endoderm differentiation cultures. C. Anti-H3K27me3 ChIP-qPCR analysis on regulatory regions of primitive streak genes (*T* and *EOMES*) and endodermal genes (*FOXA2* and *SOX17*) in CHOPWT2.1 iPS cell endoderm differentiation cultures on D0 and D1. D. Anti-SUZ12 ChIP-qPCR on neuroectoderm gene loci in H9 hES cell differentiation cultures at D0 and D1. E. (left panel) *EZH2* and *SUZ12* qRT-PCR expression profiles in H9 and CHOPWT2.1 differentiation cultures on D0, D1 and D2. (right panel) EZH2 and SUZ12 intracellular flow cytometric analysis on H9 hES cells at D0 and D1. F. (left panel) Anti-EZH2 and anti-H3K27me3 ChIP-qPCR on D1 endoderm differentiation cultures of H9 ES cells treated with and without Chir. (right panel) EZH2 and SUZ12 intracellular flow cytometry on D1 endoderm differentiation cultures of H9 ES cells treated with and without Chir. Data shown are from three independent experiments. Statistical significance represented as * for $p < 0.05$.G. Flow cytometry analysis of OCT4, NANOG, SOX2 and EOMES on D1 of CHOPWT2.1 iPS cell differentiation cultures with and without Chir. H. Anti-H3K27me3 ChIP-qPCR in CHOPWT2.1 iPS cell differentiation cultures on D1 with and without Chir. Sites used for ChIP-qPCR are indicated in kilobases (K) from the transcription start site. Data shown are from three independent experiments. Statistical significance indicated as * for $p < 0.05$.

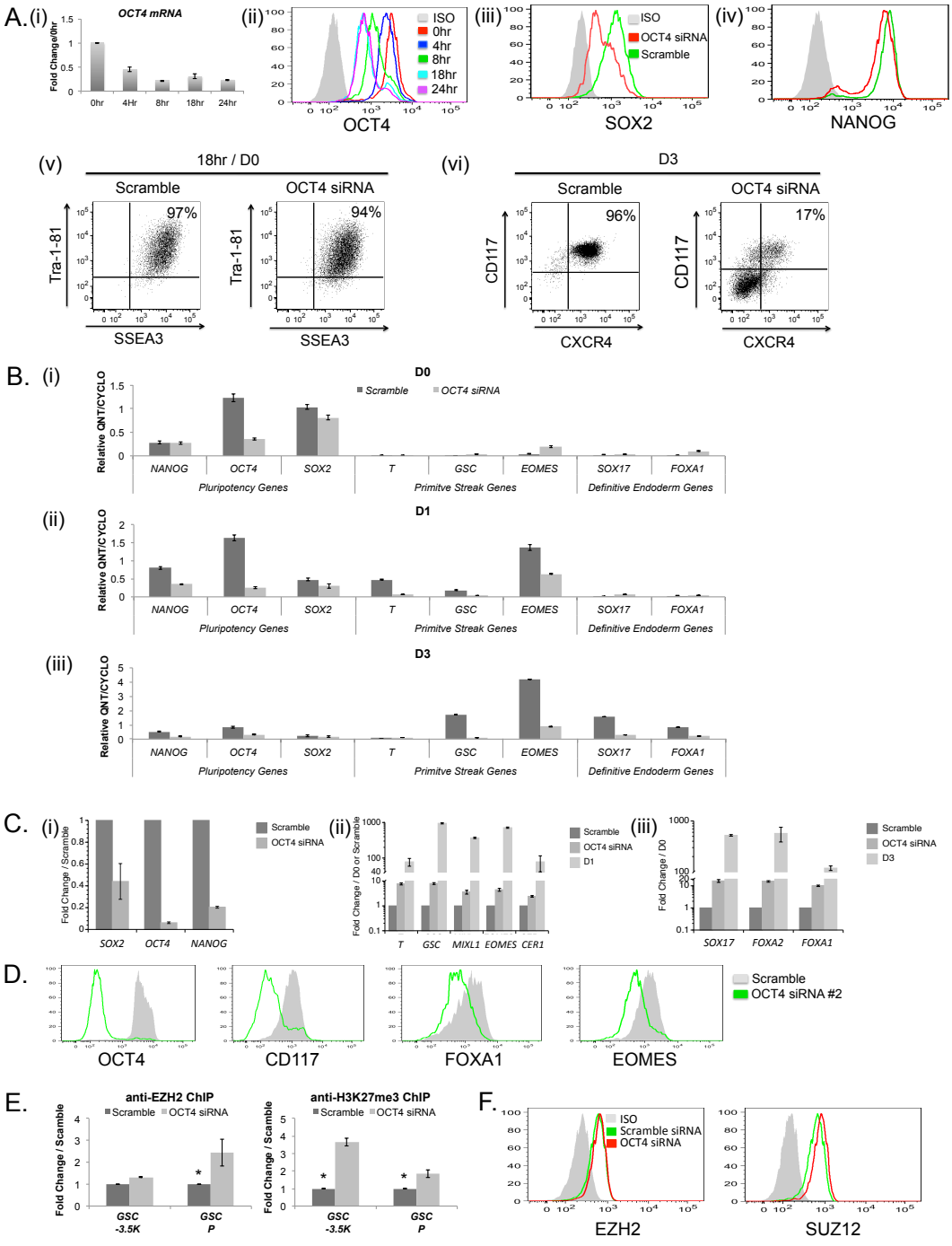
A.
(i)
OCT4 mRNA
Fold Change/0hr
0hr
4Hr
8hr
18hr
24hr
(ii)
ISO
0hr
4hr
8hr
18hr
24hr
OCT4
(iii)
ISO
OCT4 siRNA
Scramble
SOX2
(iv)
NANOG
(v)
18hr / D0
Scramble
OCT4 siRNA
97%
94%
Tra-1-81
SSEA3
(vi)
D3
96%
17%
CD117
CXCR4
B.
(i)
D0
Scramble
OCT4 siRNA
Relative QNT/CYCLO
NANOG
OCT4
SOX2
T
GSC
EOMES
SOX17
FOXA1
Pluripotency Genes
Primitve Streak Genes
Definitive Endoderm Genes
(ii)
D1
(iii)
D3
C.
(i)
Fold Change / Scramble
SOX2
OCT4
NANOG
(ii)
Fold Change / D0 or Scramble
D1
T
GSC
MIXL1
EOMES
CER1
(iii)
Fold Change / D0
D3
SOX17
FOXA2
FOXA1
D.
OCT4
CD117
FOXA1
EOMES
Scramble
OCT4 siRNA #2
E.
anti-EZH2 ChIP
anti-H3K27me3 ChIP
Fold Change / Scramble
GSC -3.5K
GSC P
F.
ISO
Scramble siRNA
OCT4 siRNA
EZH2
SUZ12

Figure S2. Gene expression profile and ChIP-qPCR after *OCT4* knockdown. A. (i) QRT-PCR and (ii) Flow cytometry time course of OCT4 siRNA knockdown in H9 ES cells. (iii-vi) Flow cytometry analysis of pluripotency markers (iii) SOX2, (iv) NANOG, (v) SSEA3 and TRA-1-81 on H9 hES cells transfected with scramble or *OCT4*-siRNA at D0 (18 hours after transfection) and (vi) definitive endoderm markers, CXCR4 and CD117, on D3 endoderm differentiation cultures. B. QRT-PCR gene expression analysis of H9 ES cells 72 hrs after *OCT4* siRNA knockdown (i) pluripotency genes, (ii) primitive streak genes comparing to D1 differentiation cells and (iii) definitive endoderm genes comparing to D3 differentiation cells. C. QRT-PCR gene expression analysis of endoderm differentiation cultures at (i) D0, (ii) D1 and (iii) D3 after *OCT4* knockdown. D. A second *OCT4* siRNA (#2) can also knockdown *OCT4* and impair endoderm differentiation. Flow cytometry analysis on OCT4 and endodermal markers in differentiation cultures as described in figure 3. E. ChIP-qPCR analysis of anti-EZH2 and anti-H3K27me3 at *GSC* regulatory regions after *OCT4* knockdown at D1 of endoderm differentiation cultures. Sites used for ChIP-qPCR are indicated in kilobases (K) from the transcription start site. Data shown are from three independent experiments. Statistical significance indicated as * for $p < 0.05$. F. EZH2 and SUZ12 intracellular flow cytometric analysis on H9 D1 OCT4 knockdown cells.

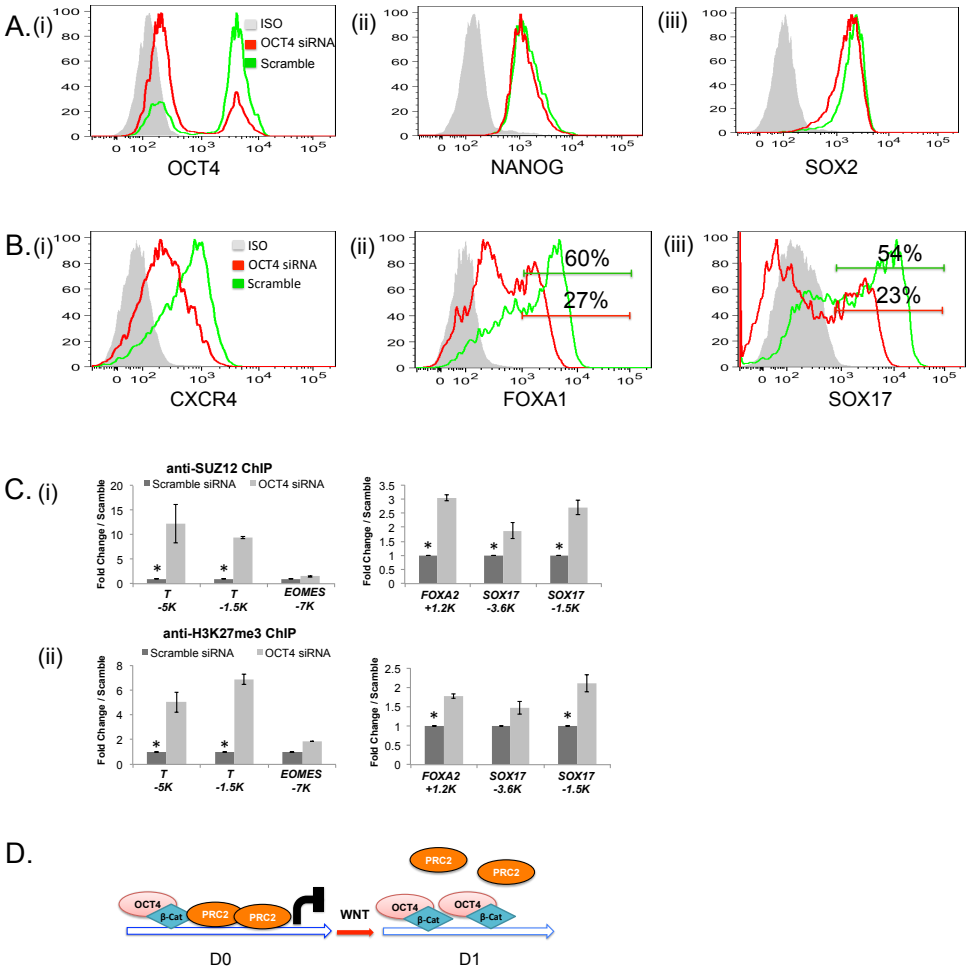
A.(i)
ISO
OCT4 siRNA
Scramble
OCT4
(ii)
NANOG
(iii)
SOX2
B.(i)
ISO
OCT4 siRNA
Scramble
CXCR4
(ii)
60%
27%
FOXA1
(iii)
54%
23%
SOX17
C. (i)
anti-SUZ12 ChIP
Scramble siRNA
OCT4 siRNA
Fold Change / Scramble
T -5K
T -1.5K
EOMES -7K
FOXA2 +1.2K
SOX17 -3.6K
SOX17 -1.5K
(ii)
anti-H3K27me3 ChIP
D.
PRC2
OCT4
β-Cat
WNT
D0
D1

Figure S3. Analysis of *OCT4* knockdown in CHOPWT2.1 iPS cells.
CHOPWT2.1 iPS cells were transfected with siRNA against *OCT4* or a control scramble siRNA construct 18 hours prior to initiation of endoderm differentiation as described in Figure 1. A. Flow cytometry analysis of SOX2, NANOG and OCT4 18 hrs after *OCT4* siRNA transfection. B. Flow cytometry analysis of CXCR4, FOXA1, and SOX17 on D3 differentiation cultures treated with *OCT4* or scramble siRNA. C. (i) Anti-SUZ12 and (ii) anti-H3K27me3 ChIP-qPCR of D1 differentiation cultures treated with OCT4 or scramble siRNA on primitive streak genes (*T* and *EOMES*) and endodermal genes (*FOXA2* and *SOX17*). Sites used for ChIP-qPCR are indicated in kilobases (K) from the transcription start site. Data shown are from of three independent experiments. Statistical significance represented as * for $p < 0.05$. D. Working model of how OCT4 and β-Catenin cooperate to evict the PRC2 complex and allow endodermal gene expression.

Supplemental Tables

Table S1. siRNA Sequence

Sequence Name	Sequence
OCT4 #1 Anti-Sense	rUrArC rUrGrG rUrUrC rGrCrU rUrUrC rUrCrU rUrUrC rGrGrG rCrCrU
OCT4 #1 Sense	rGrCrC rCrGrA rArArG rArGrA rArArG rCrGrA rArCrC rArGT A
OCT4 #2 Anti-Sense	rUrCrC rArGrG rUrUrG rCrCrU rCrUrC rArCrU rCrGrG rUrUrC rUrCrG
OCT4 #2 Sense	rArGrA rArCrC rGrArG rUrGrA rGrArG rGrCrA rArCrC rUrGG A
Scramble Antisense	rArUrG rArUrA rUrArG rArCrG rUrUrG rUrGrG rCrUrG rUrUrG rUrArG
Scramble Sense	rArCrA rArCrA rGrCrC rArCrA rArCrG rUrCrU rArUrA rUrCA T

Table S2. Primers for ChIP

NAME	Forward	Reverse
EOMES -7K	GGA GAA GGC ACC CTT AAC TGG ATG T	GAT CTT GCT CTG CAC TTG CTC TGT
GSC -3.5K	CCA GTC TCA AAG CTA AGT GCT CAA ACC	TCC ACC CAC CCA ACC TCA C
GSC P	ACA GAG CAG GTG AAG AGA GAG CAA	ACA GAG CAG GTG AAG AGA GAG CAA
FOXA2 +1.2K	ACCTCGGGCTCTGCATAGTA	TTTAAACTGCCATGCACTCG
OLIGO1 P	GGCACAAGCAGCCAATGAACAC	CTTTAAACCCGGCTTGGGAACCT
PAX6 P	GCGGACTCACCTTTATGAGG	CCATTGTGGTCTTCAAGCAA
SOX17 -1.5K	CCCTAGCCAAGGCTTCACAGATACTT	CCGTCTGTCCAGTCTTGCTTATTAGTGG
SOX17 -3.6K	AGCGGTGTACTCGGCTCTTA	GCCACATGTAAAGCAGCAAA
T -1.5K	CAGGTGGTCCACTCGGTACT	AGGGAAGGTGGATCTCAGGT
T -5K	ACCAGAGAGTCCAGGGAGGT	GGCTCTCTTTCCTCATGTGC

Table S3. Primers for RT-PCR

NAME	Forward	Reverse
CER1	TCCCATACCTCCTGCTCTCACTGTTT	TGTGTCCATCTTCATGCTCCGTCTTC
CYCLO	GAA GAG TGC GAT CAA GAA CCC ATG AC	GTC TCT CCT CCT TCT CCT CCT ATC TTT ACT T
EOMES	TAT TGT CGG CTT TGC CAC AGG TCA	ACT CAA TCC CAC TGC CCA CTA CAA
EZH2	AGG AGT GTA AGC TTT GCT CTC TCT GA	CAC CGA ACA GCA GCT CCC A
FOXA2	GCA CCT TCA GGA AAC AGT CGT TGA	ACT CGT ACA TCT CGC TCA TCA CCA
FOXA1	ACT ACT CCT TCA ACC ACC CGT TCT	TAT TGC AGT GCCTGT TCG TAT GCCTTG
GSC	GGT ACT TGG TCT CCT GGA AGA GGT T	ATG CTG CCC TAC ATG AAC GTG G
MIXL1	TAG ATG TGA ACT GCC TGC CCG AA	TGT TCC TCC CAT GAG TCC AGC TTT
NANOG	CCTGAAGACGTGTGAAGATGAG	GCTGATTAGGCTCCAACCATAC
OCT4	TTC GGG CAC TGC AGG AAC AAA TTC	TAT GCA AAG CAG AAA CCC TCG TGC
SOX17	AGG AAA TCC TCA GAC TCC TGG GTT	CCC AAA CTG TTC AAG TGG CAG ACA
SOX2	ATG ACC AGCTCG CAG ACCTAC A	GGA CTT GAC CAC CGA ACC CA
SUZ12	TGT CGA AAC TTC ATG CTT CAT CTA GTC	TGT TCT TCA GTT ATT TCT TTT GCA
TBP	TTG CTG AGA AGA GTG TGC TGG AGA TG	CGT AAG GTG GCA GGC TGT TGT T

Supplemental Experimental Procedures

ChIP-qPCR Assay

ChIP assays were carried out as previously described (Tuteja et al., 2008). Briefly, cells were crosslinked with 1% formaldehyde. Ideal length of chromatin fragments (300 – 500 bp) was achieved by sonicating with Misonix3000 with a micro tip. Chromatin was immunoprecipitated with following antibodies: H3K27me3 (Millipore, 07-449), OCT4 (Santa Cruz, sc-8628), EZH2 (Active Motif, 39875), SUZ12 (Abcam, ab12073). Primer sequences are listed in Table S2.

Gene Ontology Analysis

The hES OCT4 ChIP-seq dataset (GSE21916) (Marson et al., 2008) and H3K4me3 and H3K27me3 ChIP-seq dataset (GSE23455) (Guenther et al., 2010) were used. Co-occupancy of OCT4 and bivalent domains was obtained by intersecting 2 datasets using GALAXY (Goecks et al., 2010). Then, gene ontology term enrichment was computed by feeding GREAT (McLean et al., 2010) with OCT4 binding bivalent domain position information.

Flow Cytometry and Immunofluorescence

Flow cytometry was carried out as previously described (Gadue et al., 2006). Briefly, for cell surface markers, cells were resuspended in FACS buffer and stained with anti-CXCR-4-PE (Invitrogen, MXCXCR404) and anti-CD117-APC (Invitrogen, CD11705). For intracellular markers, cells were fixed with 1.6% PFA and permeablized with saponin buffer (Biolegend). Antibodies used for intracellular staining were anti-OCT4 (Santa Cruz, sc-5279), anti-SOX2 (BD MAB2018), anti-β-Catenin (BD, 610154), anti-FOXA1 (Santa Cruz, sc-101058) and biotinylated anti-SOX17 (R&D, BAF1924). Cells were acquired with BD FACS Canto II (BD Sciences). Data were analyzed with FlowJo software.
For immunofluorescence staining, cells were replated onto matrigel-coated glass cover slips. Cells were harvest at D0, D1 and D2 of differentiation and fixed with 1% PFA. Cells were permeablized with 0.1% Triton-X 100. Antibodies used for immunofluorescence staining were anti-OCT4 (Santa Cruz, sc-5279), anti-β-Catenin (BD, 610154). Stained cells were mounted with ProLong Gold anti-fade mountant with DAPI (Invitrogen). The stained cells were visualized using a fluorescence microscope (Leica DMI 4000B) and images captured and analyzed using the Leica Application Suite software. Distribution of fluorescence intensity across a single representative cells was measured and plotted, demonstrating nuclear localization of ABC at D1.

Reverse Transcription and Real-time Quantitative PCR (qRT-PCT)

RNA was extracted using RNeasy Micro RNA Extraction kit (Qiangen). Reverse transcription was performed using SuperScript III (Invitrogen), as per manufacturer instructions. Quantitative PCR was performed on a Light Cycler 480 (Roche) using LightCycler SYBR Green Mater Mix I (Roche) and normalized to the housekeeping genes TBP or CYCLOPHILIN. Data shown are representative of three independent experiments. Statistical significance indicated as * for $p < 0.05$. Primer sequences are listed in Table S3.

Western Blot and Immunoprecipitation

Western blot and immunoprecipitation were carried out as previously described (Kelly et al., 2011). For immunoprecipitation, 20 million of D0 hESCs or D1 cells were treated with hypotonic buffer for 10 min on ice, then, nuclei protein was extracted with high salt buffer. 400 µg of nuclei protein was subjected to immunoprecipitation with anti-OCT4 antibody (Santa Cruz, sc-8628). Eluted material was blotted with either anti-OCT4 antibody (Santa Cruz, sc-8628), anti-totalβ-catenin antibody (BD) or anti-activatedβ-catenin antibody (Life Sciences, 712700).

Statistics

All experiments were performed at least three times and statistical significance was calculated by Student's t test. For ChIP qPCR results, *p* value was calculated by Student's T test. Error bars represent SEM from 3 biological replicates. All experiments were performed at least three times. Results with statistic significance ($p<0.05$) were labeled with asterisk (*).

Supplemental References

Goecks, J., Nekrutenko, A., Taylor, J., and Galaxy, T. (2010). Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. Genome biology *11*, R86.

Guenther, M.G., Frampton, G.M., Soldner, F., Hockemeyer, D., Mitalipova, M., Jaenisch, R., and Young, R.A. (2010). Chromatin structure and gene expression programs of human embryonic and induced pluripotent stem cells. Cell Stem Cell *7*, 249-257.

Kelly, K.F., Ng, D.Y., Jayakumaran, G., Wood, G.A., Koide, H., and Doble, B.W. (2011). beta-catenin enhances Oct-4 activity and reinforces pluripotency through a TCF-independent mechanism. Cell Stem Cell *8*, 214-227.

Marson, A., Levine, S.S., Cole, M.F., Frampton, G.M., Brambrink, T., Johnstone, S., Guenther, M.G., Johnston, W.K., Wernig, M., Newman, J.*, et al.* (2008). Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. Cell *134*, 521-533.

McLean, C.Y., Bristor, D., Hiller, M., Clarke, S.L., Schaar, B.T., Lowe, C.B., Wenger, A.M., and Bejerano, G. (2010). GREAT improves functional interpretation of cis-regulatory regions. Nature biotechnology *28*, 495-501.