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Supplemental Data

An Extended Transcriptional Network

for Pluripotency of Embryonic Stem Cells

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

ES cell lines and culture

Mouse J1 ES cell lines were maintained in ES medium (DMEM; Dulbecco's modified Eagle's medium) supplemented with 15% fetal calf serum, 0.1 mM ß-mercaptoethanol, 2 mM L-glutamine, 0.1 mM non-essential amino acid, 1% of nucleoside mix (100X stock, Sigma), 1000U/ml recombinant leukemia inhibitory factor (LIF; Chemicon) and 50 U/ml Penicillin/Streptomycin. ES cells expressing biotin-tagged Nanog, Dax1, Nac1, Oct4, Zfp281, and Rex1 were described previously (Wang et al., 2006). For biotin-tagged Klf4, Sox2 and c-Myc, cDNAs for each gene were amplified via PCR from an embryonic cDNA library (Clontech) and incorporated into the pEF1α-FLBIO vector. Each vector was stably transfected into the ES cells stably expressing *Escherichia coli* biotin ligase BirA enzyme under the control of EF1α promoter. Positive clones were selected by growth in Puromycin and the level of ectopic expression of transcript or protein was detected by quantitative RT-PCR or Western blotting assay with anti-streptavidin-HRP and factor specific antibodies (Wang et al., 2006). List of primers used for PCR validation are available in **Table S4**.

Chromatin immunoprecipitation and antibodies

For biotin-mediated ChIP, approximately, 5×10^7 mES cells expressing both BirA and biotinylated proteins were used. Briefly, cells were cross-linked by addition of final 1% formaldehyde for 7 min at room temperature. Cross-linking was terminated by adding final 125 mM glycine and cells were washed with cold phosphate-buffered saline (PBS) containing PMSF, scraped off the plates, collected by centrifugation and washed again. Collected cell pellet was resuspended in SDS ChIP buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl pH 8.1, 150 mM NaCl, and protease inhibitors). Cells were sonicated and fragmented DNA was visualized on an agarose gel (average size 0.5-1 kb). The sample was centrifuged at 12000 rpm at 4°C for 10 min and supernatant

was collected. Sample was pre-cleared with protein A beads at 4°C for 1 hr and incubated with streptavidin beads (Dynabeads® MyOne™ Streptavidin T1) at 4°C overnight. For reference sample, J1 ES cells expressing BirA enzyme without biotinylated protein were used. Immunoprecipitated complexes were successively washed with buffer I (2% SDS), buffer II (0.1% Deoxycholate, 1% Triton X-100, 1 mM EDTA, 50 mM HEPES pH 7.5, 500 mM NaCl), buffer III (250 mM LiCl, 0.5% NP-40, 0.5% Deoxycholate, 1 mM EDTA, 10 mM Tris-Cl pH 8.1) and TE buffer (10 mM Tris-Cl pH 7.5, 1 mM EDTA). All washes were for 8 min at room temperature. SDS elution buffer was added and incubated at 65°C overnight to reverse crosslink protein-DNA complexes. The sample was treated with RNase A and Proteinase K, extracted with phenol:chloroform and precipitated. The pellet was resuspended in 25μl of water.

Conventional ChIP reaction was performed as described previously (Kim et al., 2005) with 1:100 dilution of following antibodies; anti-Nanog (ab21603, Abcam), anti-c-Myc (sc-764x, Santa Cruz), anti-H3K4me3 (ab8580, Abcam) and anti-H3K27me3 (07-449, Upstate). Input genomic DNA was used for the reference sample. For western blotting assays shown in **Figure S2**, anti-Nanog, anti-Sox2, anti-Dax1, anti-Oct4, anti-Klf4, and anti-c-Myc antibodies were purchased from Santa Cruz Biotechnology. Anti-Nac1 was a generous gift from Scott Mackler (University of Pennsylvania School of Medicine).

Microarray and data processing

At least three biological replicates of hybridization were performed on Affymetrix GeneChip Mouse promoter 1.0R arrays. ChIP samples were amplified by ligation-mediated PCR (LM-PCR), as described previously (Ren et al., 2000). Subsequent DNA fragmentation and biotin labeling steps were performed according to the manufacturer's instructions
(http://www.affymetrix.com/support/downloads/manuals/chromatin_immun_ChIP.pdf). Microarray hybridization, washing, and scanning were performed at Microarray Core Facility - Dana-Farber Cancer Institute. MAT (Model-based Analysis of Tiling-array) was applied to predict the target loci (Johnson et al., 2006), and targets were predicted at the MAT with p-value=1.00E-6. Genomic regions between 8 kb upstream and 2 kb downstream of transcription start site (TSS) of well annotated genes from mouse genome annotation released in March 2006 (mm8) was used, and for the genes that have multiple transcripts with same TSS presented in RefSeq, we used information from only one transcript the analysis (total 19,253 genes).

For gene expression analysis, we obtained expression data from a previous study (Perez-Iratxeta et al., 2005) where a triplicate time course experiment of *in vitro* differentiation of mES cells was performed (11 time points; day 0, 6 hours, 12 hours, 18 hours, 24 hours, 36 hours, 48 hours, 4 days, 7 days, 9 days, and 14 days). The CEL files were imported into dChIP software(Schadt et al., 2001) for data normalization, extraction of expression values, and generating GTC file for GSEA analysis (Subramanian et al., 2005).

For the supervised clustering image shown in **Figure 4A**, we first performed un-supervised hierarchical clustering across the transcription factors based on their target correlation using Cluster software (**Figure 3E and Figure 4A, cluster)** (Eisen et al., 1998). To get a simple view of common targets of multiple factors and unique targets of single factor, we first randomized the order of 6632 genes, then sequentially sorted targets of each factor from Myc (the factor that has the most different set of targets as shown in Figure 2D hierarchical cluster) to Nanog. For visualization of target gene expression profile in **Figure 4A**, expression values of each gene during ES cell differentiation time course of 0 hours, 6 hours, 12 hours, and 18 hours (EX: 0-18h; red line), and day 4, day 7, day 9, and day 14 (EX: 4-14d; blue line) were averaged based on the similarity of expression profiles before moving window average was applied. Examples of the individual time point data (0 hours, 12 hours, 36 hours, 48 hours, 9 days, and 14 days) are shown in **Figure S8**.

Quantitative real-time PCR

Quantitative PCR was performed with a Bio-Rad iCycler in a 25µl SYBR Green reaction with approximately 2% of ChIP sample. PCR parameters were: 95°C for 3 min and 40 cycles of 95°C 20 sec, 60°C 30 sec, and 68°C 30 sec. The amount of each amplification product was determined relative to a standard curve, and fold enrichment was calculated by comparison of amplified product from bioChIP sample and ChIP samples from BirA containing ES cells. Primer pairs for quantitative ChIP-PCR were designed using ±150bp genomic sequence information specific to the predicted target loci to generate 100bp to 125bp amplified products. All primer sequences used in **Figure 1B** and **Figure S5** are listed in **Table S4**.

Visualization of Regulatory Network

Cytoscape software version 2.3 (Shannon et al., 2003) was used for the visualization of transcription regulatory networks shown in **Figure 6**.

SUPPLEMENTAL REFERENCES

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SUPPLEMENTAL FIGURES

Figure S1. Comparison of bioChIP-chip and antibody ChIP-chip

Global patterns of Nanog and Myc binding to the target promoters of genes on chromosome 8 are displayed using Affymetrix Integrated Genome Browser and screen captured. 'bioNanog', and 'bioMyc' represent the data from bioChIP-chip. 'Nanog antibody', and 'Myc antibody' represent the data from antibody ChIP-chip.

Figure S1

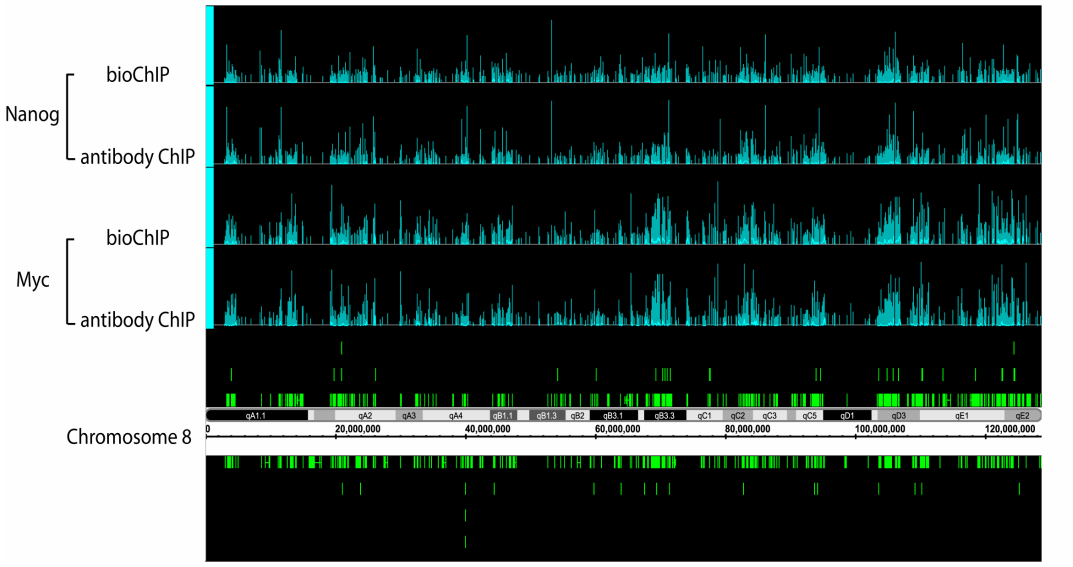


Figure S2. Sub-endogeneous expression of biotin tagged proteins in biotin-tagged cell lines

Nuclear extracts from ES cells expressing each biotin-tagged protein were subjected to Western blot analyses using the indicated antibodies. Both the biotin-tagged and endogenous levels of the protein are shown from bioTF cells but BirA expressing cells express only endogeneous proteins. n.s indicates non-specific.

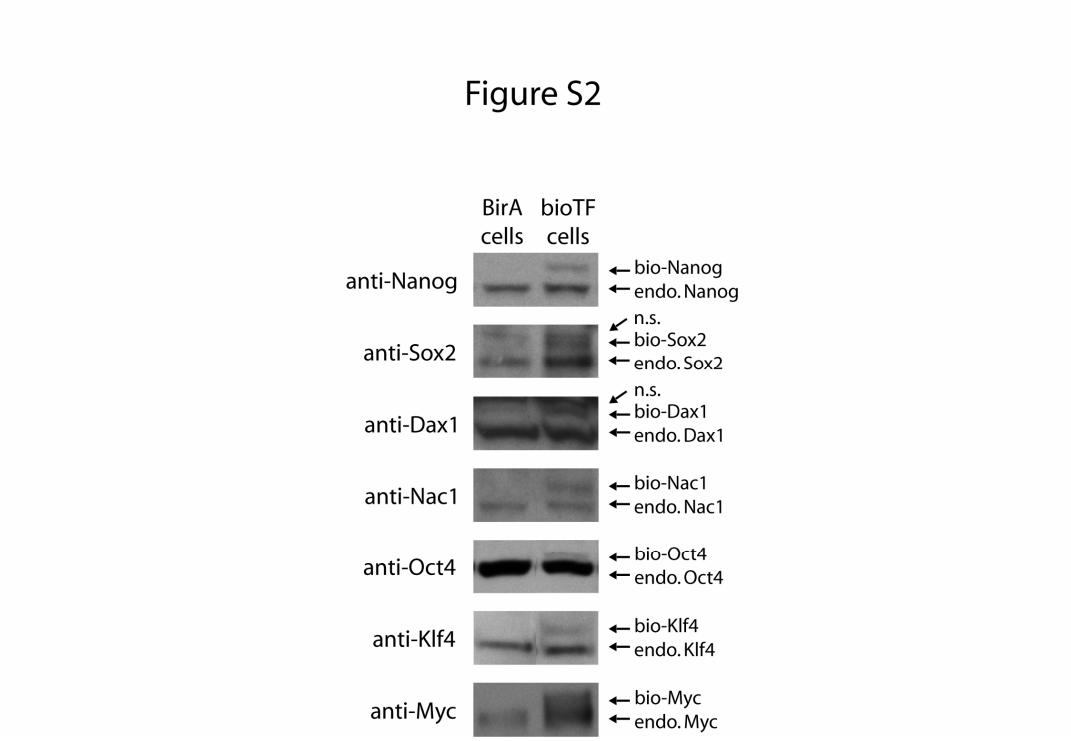


Figure S3. Correlation of Nanog occupancy among different cell lines based on target peak similarity shown in **Figure 2.**

Figure S3

		bioNanog	J1ES	BirA	bioNanog	bioOct4	bioDax1
bioChIP/BirA	bioNanog	1.000					
Nanog antibody ChIP/input	J1ES	0.896	1.000				
	BirA	0.883	0.984	1.000			
	bioNanog	0.895	0.985	0.970	1.000		
	bioOct4	0.886	0.964	0.967	0.962	1.000	
	bioDax1	0.883	0.967	0.973	0.963	0.968	1.000

Figure S4. Representative examples of target promoter occupancy by each factor
Multiple factors bind to their common target promoters in close proximity (Sox2 and Evx1). Some targets are co-occupied by fewer factors (Fgfr2 and Cdx4). Examples of Rex1 and Myc common targets are also shown (Crat/Ppp2r4 and Anp32b).

Figure S4

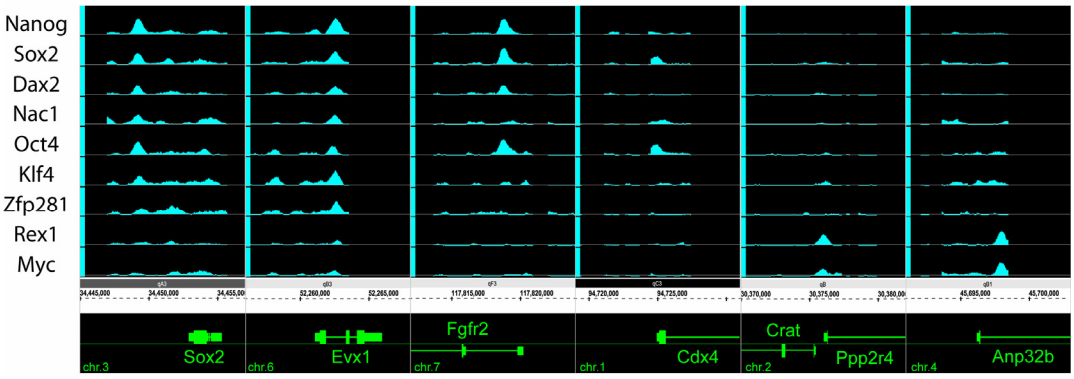


Figure S5. Target validation by quantitative PCR

Predicted target loci of each factor were validated by site-specific PCR analysis. Predicted targets and non-targets are separated in different colors (black and grey bars, respectively). Y-axis represents a relative fold enrichment of predicted target loci tested from three independent bioChIP reactions over reference samples from BirA expressing cell and normalized to Gfi1b. Error bars represent standard deviation. Primers used in this study are listed in **Table S4**.

Figure S5

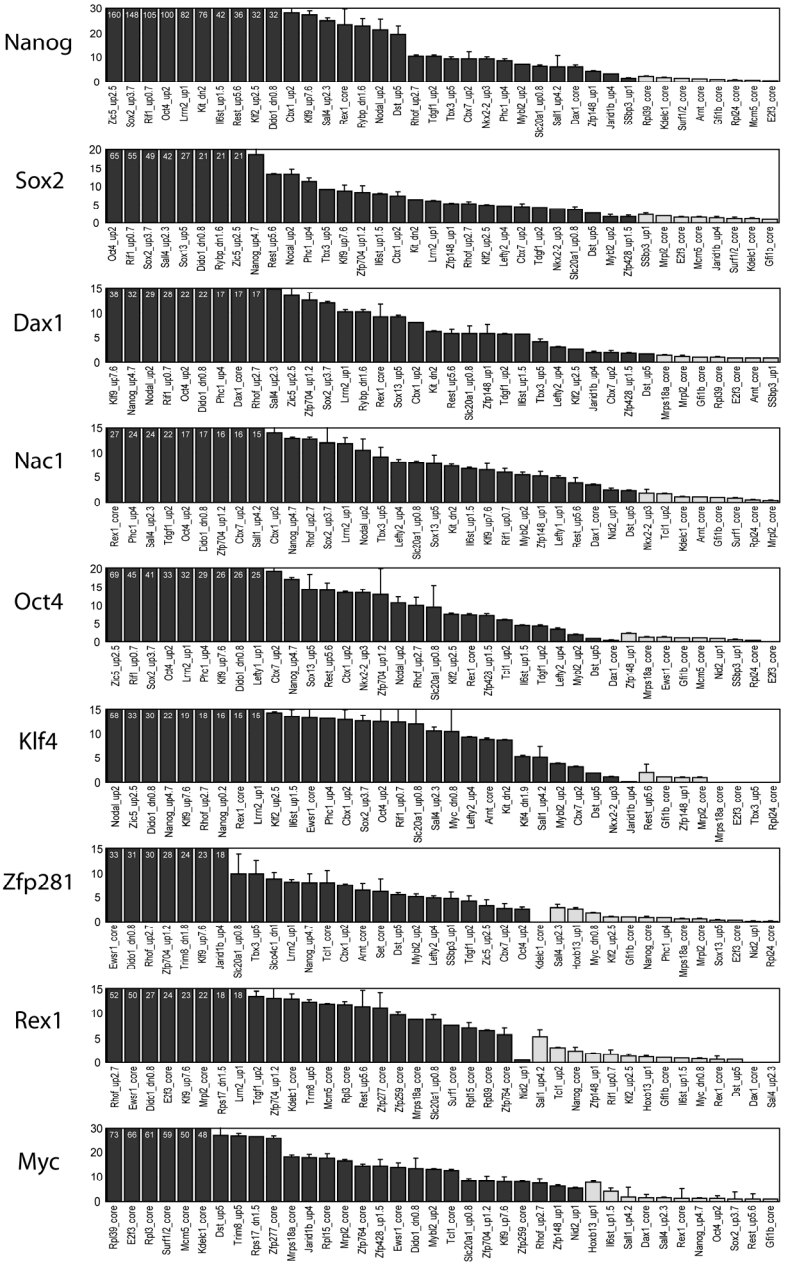


Figure S6. Functional classification of targets of each factor

Percent of gene hit against total number of function hits for targets of each transcription factor was calculated from PANTHER (www.pantherdb.org). The obtained percent value was divided by the value calculated for all mouse genes, and multiplied by 100. Values above 100 indicate enrichment and values below 100 indicate depletion for each GO term. Targets of Myc or Rex1 are implicated in protein metabolism, whereas targets of the other factors are in developmental processes.

Figure S6

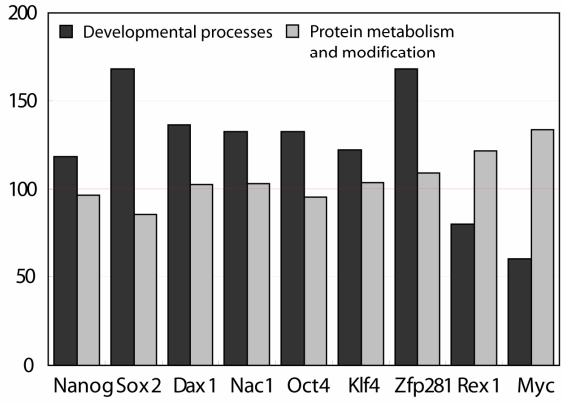


Figure S7. Cluster of genes not occupied by any of nine transcription factors

(A) Schematic representation of whole genome distribution of H3K4me3, H3K27me3, and nine factors. X-axis represents all RefSeq genes based on their chromosomal positions. Predicted histone marks H3K4me3 (red), H3K27me3 (blue) and transcription factor binding (green) on the promoters of each gene were initially assigned 1 (presence) or 0 (absence). Moving window average (bin size 100 and step size 1) was applied across the genes. Red dots represent some clusters of genes devoid of any of the nine transcription factor occupancy, H3K4me3 and H3K27me3 marks on their promoters.

(B) Enlarged view of chromosome 2 containing a cluster of olfactory receptor genes.

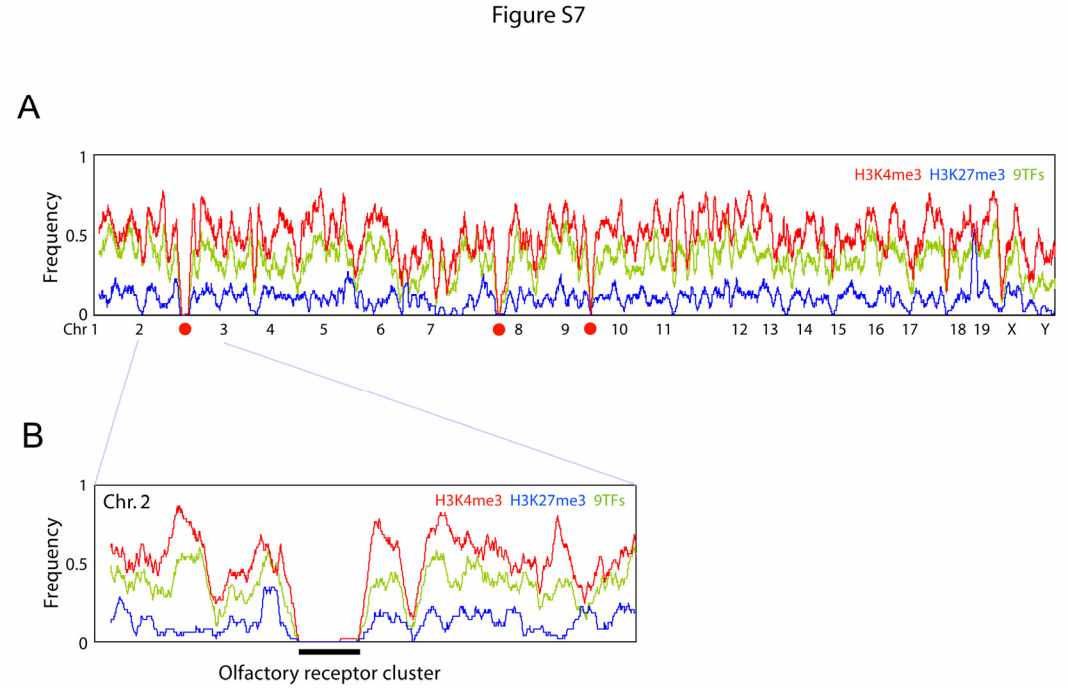


Figure S8. Transcription factor occupancy to the target promoter and corresponding gene expression during differentiation time course

Extension of analysis shown in **Figure 4A**. Instead of averaging multiple time points, 6 different time points are presented in three different columns showing overall expression profiles between earlier time points (0h and 12h) or later time points (9d and 14d) are similar.

Figure S8

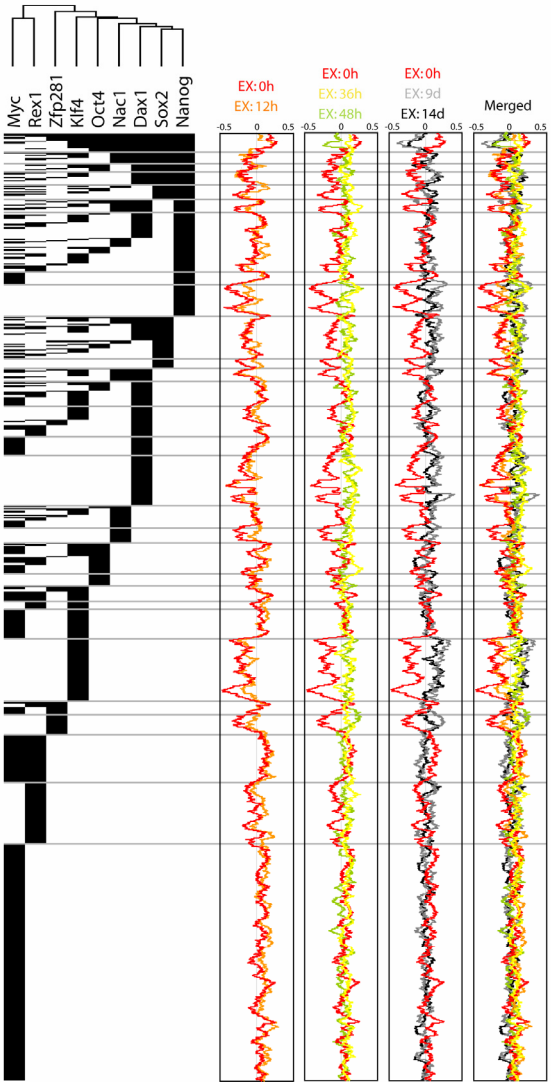


Figure S9. Target gene expression and transcription factor occupancy

Extension of analysis shown in **Figure 4**. Target promoters were classified based on the number of co-occupying factors onto the promoters and corresponding gene expression upon differentiation was tested using GSEA software.

Figure S9

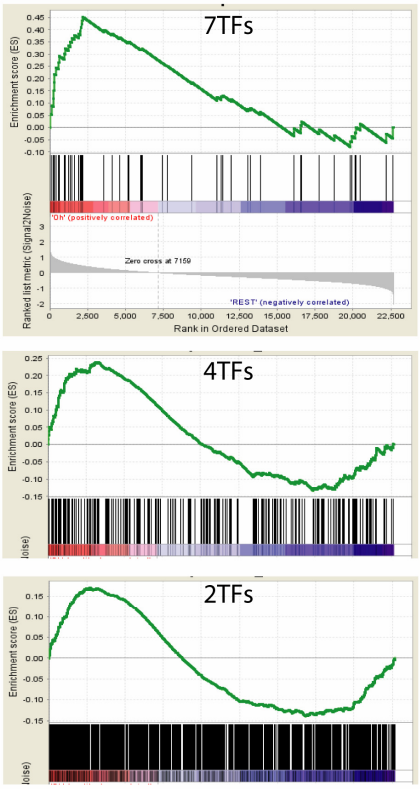
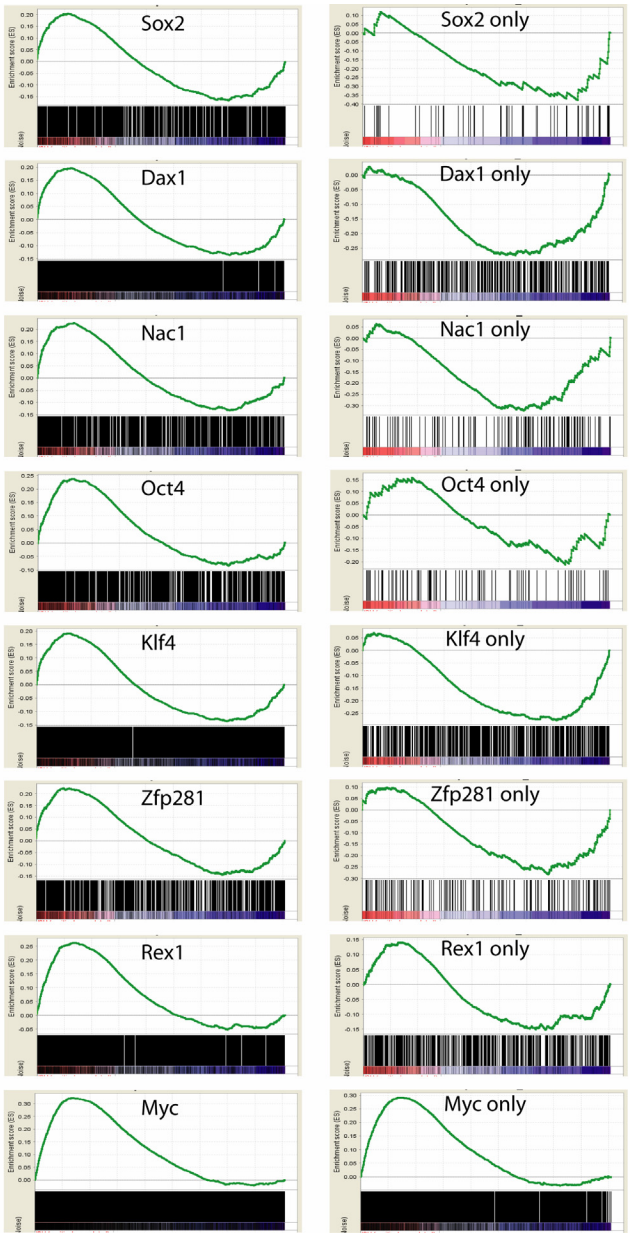


Figure S10. Single factor only targets are inactivated or repressed in ES cells

Extension of analysis shown in **Figure 4F and 4G**. Targets of eight factors were tested in two different ways using GSEA software. Figures shown on the left column represent all the targets of each factor and their gene expression upon ES cell differentiation. For figures shown on the right column (depicted as factor-'only') the subset of targets predicted to be occupied by only one factor were used.

Figure S10



SUPPLEMENTAL TABLE

Table S4. Primer sequences for RT-PCR and ChIP-PCR

PrimerName	Gene	Approximate distance from TSS	Forward sequence	Reverse sequence	Application
Arnt_core	Arnt	core	CTAATCTGCGGAGTGGCTCTT	CCTCACCTGGGTTAGCTGTAGT	ChIP-PCR
Cbx1_up2	Cbx1	upstream 2kb	GCACCCTGAATTCCTTTCTTTAT	AATCGTAACTCCAGGACATCTGA	ChIP-PCR
Cbx7_up2	Cbx7	upstream 2kb	TTATTTAGAAGGGGCTTCCTTTG	GGGTGTTTGTAAAGGAGGGATAG	ChIP-PCR
Dax1_core	Dax1	core	GTTAATGGCAAGAGTTGGAACAG	ACCACATACCACCTTTCCTTCTT	ChIP-PCR
Dido1_dn0.8	Dido1	downstream 0.8kb	GGAAGTTGGAGGCTCTTTAACAC	AATGACTGAATTTGCATTCACCT	ChIP-PCR
Dst_up5	Dst	upstream 5kb	CTGTTTGACTCTGCTTTCTGGTT	AGGTTGCCTTGATGGTTAGTGTA	ChIP-PCR
E2f3_core	E2f3	core	GCGGAGATATGCAAATATGGTT	CTGCTGCTGACAATGAATGAAG	ChIP-PCR
Ewsr1_core	Ewsr1	core	TTCAGAGACGGTCCCTAGAGC	GACGTGACGAGTGGTCTAAAAA	ChIP-PCR
Gfi1b_core	Gfi1b	core	CGCCAGATTTTGACACAAATAA	CTGCACAGACAGACACTTCTCC	ChIP-PCR
Hoxb13_up1	Hoxb13	upstream 1kb	CTTAACTTTCAACTTGGCCTTGA	GGAGGAGTCTGTAGCCTTAGAGC	ChIP-PCR
Il6st_up1.5	Il6st	upstream 1.5kb	ATGGGAAGTAACAACCGTAGGAT	AAGACTTGTAAGGGCCTTTGTTC	ChIP-PCR
Jarid1b_up4	Jarid1b	upstream 4kb	CACCGAGTGAGACGACTAGATTC	AAATATTTGTAGAAAGGGCCTTGA	ChIP-PCR
Kdelc1_core	Kdelc1	core	CAGGATTTCCGCTAGTCCTATG	GTCTTCTGCTAGTGGATGAGTGG	ChIP-PCR
Kit_dn2	Kit	downstream 2kb	ATTTAAATTGGGAGTGGGTGTGT	TGCTTTCTGAAATTGTGAACTCC	ChIP-PCR
Klf2_up2.5	Klf2	upstream 12.5kb	ACTTCAGCTCACTCCCCCTACT	AATAGATCAGTAGCTCAGAGCCAGA	ChIP-PCR
Klf4_dn1.9	Klf4	downstream 1.9kb	CTCCTCTACAGCCGAGAATCTG	AGGAGCTCAGCCACGAAG	ChIP-PCR
Klf9_up7.6	Klf9	upstream 7.6kb	TTAAAATGCCCTTGTTGTTTCTG	CCCATTTTAAAAACCACCTCTTC	ChIP-PCR
Lefty1_up1	Lefty1	upstream 1kb	AGCTGCTCTTCTGCATAACAAA	AGACAATCTTAGTCGGGGGATAG	ChIP-PCR
Lefty2_up4	Lefty2	upstream 2kb	CAAGTGTTTGTATTTTGCCACAG	CAGACTTCTTTGAGGTTGCAGAG	ChIP-PCR
Lrrn2_up1	Lrrn2	upstream 1kb	ATTAAGGGGCCAACTCTGTCTAT	GGCTTCTGGGTTCTGTTTATTCT	ChIP-PCR
Mcm5_core	Mcm5	core	GCATTTTCTACAGCGACAGCTT	CACTCGGTACTGTCTCAGGAACT	ChIP-PCR
Mrpl2_core	Mrpl2	core	ATTGTTTTGACGGCTCCAAG	CTTGCCTCTCCAAAGAACAAGT	ChIP-PCR
Mrps18a_core	Mrps18a	core	CGAAAGTCAAGGAGAACTGAGAG	GGGAGAATTAACACTCAGCCTTT	ChIP-PCR
Mybl2_up2	Mybl2	upstream 2kb	AAGGAATAGCCTTGATTTGGAAG	CTCAGAACGGATAACAGAATTGC	ChIP-PCR
Myc_dn0.8	Mycd	downstream 0.8kb	ACAGTCTTTCTTCCATTCCTGTG	TGTCATCTTGACAAGTCGCTCTA	ChIP-PCR
Nanog_up0.2	Nanog	core	TGGGGTAAACTTAAGGCTATGG	AGCTCTAAGCCGGTTCTCATTT	ChIP-PCR
Nanog_up4.7	Nanog	upstream 4.7kb	AATGAGGTAAAGCCTCTTTTTGG	ACCATGGACATTGTAATGCAAA	ChIP-PCR
Nid2_up1	Nid2	upstream 1kb	CCCCATGGGTCTTTAAGAATTAC	GGCTCCTTAGAAGCAAAGGTAGT	ChIP-PCR
Nkx2-2_up3	Nkx2-2	upstream 3kb	AAGGGTGACGAACAGAACTCTC	GACTAGGAGGGCAAGAGAAACTC	ChIP-PCR

Nodal_up2	Nodal	upstream 2kb	TGGGGACACATCCTACTAGGTAA	TCAGAAGTGGAATTTGGAGAGAG	ChIP-PCR
Phc1_up4	Phc1	upstream 4kb	AGGTTGGGGAAATAAGGACATAA	GGGCTTCTATAGGAAAACTCAACA	ChIP-PCR
Rest_up5.6	Rest	upstream 5.6kb	GTCACACTGAGGAAATGAAGGAC	CCCAGTCTCTGAAACAGAAAAGA	ChIP-PCR
Rex1_core	Rex1	core	GCATCCTCTGCTTGTGTAAATTC	CTCAGTTATGCAAATGCCTCTTC	ChIP-PCR
Rhof_up2.7	Rhof	upstream 2.7kb	GAGGGAAAGTTGAACAAAAACCT	ACCTAGGGTCCTAACCTGTCACT	ChIP-PCR
Rif1_up0.7	Rif1	upstream 0.7kb	CGTTGTTTAAGGGTTTACTGTCG	TGCCTAGAATTGGTTTACTTCCA	ChIP-PCR
Rpl15_core	Rpl15	core	AGAAAGCCTTCTTCCTTTCCTCT	GGTCGGATGGCTGAAGACTA	ChIP-PCR
Rpl24_core	Rpl24	core	GAGGGAATATGGATGGTGTGTAA	AAACTCAAAGAAATCTGGCTCTG	ChIP-PCR
Rpl3_core	Rpl3	core	TGGGTGTATTTTTCAGGGTTTTA	GTTCTCATCCTGCAAGGGTAAAT	ChIP-PCR
Rpl39_core	Rpl39	core	ATCAGCGTAATCCTAAGGGACTT	AACCAATGCATTCCTAAAGACCT	ChIP-PCR
Rps17_dn1.5	Rps17	downstream 1.5kb	GCTTTCCTCCCTGGTTATTTCTA	GCCTATCCCACCTTCTCATACTT	ChIP-PCR
Rybp_dn1.6	Rybp	downstream 1.6kb	GCAATTGGAGATATGTTCTGGAG	CAACAATTCACACACTTCACCAT	ChIP-PCR
Sall1_up4.2	Sall1	upstream 4.2kb	CCCAAGCCCTAAGTCTAGAAAAG	TTAACCACCTGGGGTTTATTTGT	ChIP-PCR
Sall4_up2.3	Sall4	upstream 2.3kb	CTGCTCAAGGCATTGTAAGCTAT	AGACAGAAGTGGAAGAAGCCTTT	ChIP-PCR
Set_core	Set	core	GAGGCAACAGCACGTATTAAGTC	AGAAACCCTGCCTTACACTGAG	ChIP-PCR
Slc20a1_up0.8	Slc20a1	upstream 0.8kb	TGTGCTTTACACACAATTCCAAC	CTTGAACAAACCCTAGGTCTCCT	ChIP-PCR
Slco4c1_dn1	Slco4c1	downstream 1kb	CTCCTTTGGGAAAACGATAAGTC	GCCTTTAATCCTCCCTCTGTAAA	ChIP-PCR
Sox13_up5	Sox13	upstream 5kb	TTTTGTCTGAAGTTCCTGAGAGG	GCTGAGGGCAACAATTACATAAC	ChIP-PCR
Sox2_up3.7	Sox2	upstream 3.7kb	GCAATGCTGAGAAATTCCAGTT	GTTCCCCTCCTCTCCTAATCTC	ChIP-PCR
SSbp3_up1	SSbp3	upstream 1kb	AGACAGTGCCGACAATGTTTTAT	TCCAAATCAAAAACTGGTGAGAT	ChIP-PCR
Surf1/2_core	Surf1/2	core	CAAGAAGTACCAACGGCTGTC	GCCAAACTACGTACCGATTCTTT	ChIP-PCR
Tbx3_up5	Tbx3	upstream 5kb	GACCATTGTCTAGCTCAATCACC	TCCTGTAAGGCATTTCTTCTCTG	ChIP-PCR
Tcl1_core	Tcl1	core	AGGACAGACTGAAGGTGACAGAG	TCAGCTACTTTGTGTGACTGCTC	ChIP-PCR
Tcl1_up2	Tcl1	upstream 2kb	CCCCTTTGTGGAATTTACATTTT	TTCTTGAGAGGTGGTGTTTCATT	ChIP-PCR
Tdgf1_up2	Tdgf1	upstream 2kb	AGTGTGGACAAGTCCTGAGAGAG	TGGTAAATAACTGAGCCCTGAAA	ChIP-PCR
Trim8_dn1.8	Trim8	downstream 1.8kb	CCCTCCCATTGTATACACCCTAC	AAGTATGCCCCACTCCAGTTC	ChIP-PCR
Trim8_up5	Trim8	upstream 5kb	CTGCCTCTAGCAAACAGAGCTTA	TTTACTACTTTGCCACCCTTCTG	ChIP-PCR
Zfp148_up1	Zfp148	upstream 1kb	CTCTCAGGAAGTTCCCAATAACC	AGGGAGGGCTTACAGTTAATGAC	ChIP-PCR
Zfp259_core	Zfp259	core	ACTGTTACCGGAACGTGAGTCT	GGGAGAATGACTTCAGCTTGTTT	ChIP-PCR
Zfp277_core	Zfp277	core	CCTTTCTACTGTGCCAGGTAATG	CTCACCTTCATGATCTGTGTGTC	ChIP-PCR
Zfp428_up1.5	Zfp428	upstream 1.5kb	CATTTATTGAGACCAGTGCTGTG	GAGCTCACTCTCTCCTGGTATTG	ChIP-PCR
Zfp704_up1.2	Zfp704	upstream 1.2kb	TCCAGTTAACAAGGTTTGCTCAT	TTCAGATACCACCCCAGAATTTA	ChIP-PCR
Zfp764_core	Zfp764	core	TAGGGAGCATAGTTCCTGAGATG	GGGCACCTTAATGTAGGTGAAA	ChIP-PCR
Zic5_up2.5	Zic5	upstream 2.5kb	TGTCCTTAGAAAGTCGGGATTTA	TTTTGTAGACGCCCTTTTTGTTA	ChIP-PCR
Dax1	Dax1	n/a	CAGCATCTTATACAGCTTGCTCA	CCACACTCTGGGTACAGTAGGAC	RT-PCR

Klf4	Klf4	n/a	GTGCAGCTTGCAGCAGTAAC	AGCGAGTTGGAAAGGATAAAGTC	RT-PCR
Myc	Myc	n/a	ACTACGACTCCGTACAGCCCTAT	TTTCTTCCAGATATCCTCACTGG	RT-PCR
Nac1	Nac1	n/a	TGAAGAGGACGAAGAAGAAGATG	CTGGCCAACGTTCAACATACT	RT-PCR
Nanog	Nanog	n/a	AGGGTCTGCTACTGAGATGCTCTG	CAACCACTGGTTTTTCTGCCACCG	RT-PCR
Pou5f1	Pou5f1	n/a	CTGAGGGCCAGGCAGGAGCACGAG	CTGTAGGGAGGGCTTCGGGCACTT	RT-PCR
Rex1	Rex1	n/a	CAGTCCAGAATACCAGAGTGGAA	ACTCTAGGTATCCGTCAGGGAAG	RT-PCR
Sox2	Sox2	n/a	GCGGAGTGGAAACTTTTGTC	TATTTATAATCCGGGTGCTCCTT	RT-PCR
Zfp281	Zfp281	n/a	CCCCAGAGTATGGTTATGTTCAA	GTAGAGGAGGATAACACGCACTG	RT-PCR