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Supplemental Information

Neural Stem Cell Differentiation Is Dictated by Distinct Actions of Nuclear Receptor Corepressors and Histone Deacetylases

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SUPPLEMENTARY METHODS

OPC cultures. Oli-neu cells were plated in flasks coated with 0,01% poly-L-lysine (Sigma-Aldrich) and expanded in Sato media (with 340ng/ml T3 and 400 ng/ml L-thyroxine, Sigma-Aldrich) supplemented with 1% horse serum (Invitrogen). Cells were passaged with trypsin (Invitrogen) and DNase I (Roche), and differentiated in the presence of 1mM dibutyril cAMP (Sigma-Aldrich). For lipofection or differentiation assays, Oli-neu cells were plated at the following densities: $114x10^3$ cells in 35 mm plates (Corning) or $300x10^3$ cells in 60 mm plates (Corning). For ChIP analysis, cells were expanded in 10c The postnatal OPC cells were plated in flasks coated with poly-L-ornithine and fibronectin (Sigma-Aldrich) and expanded in N1 media supplemented with FGF-2 (10ng/ml) and PDGF-BB (10ng/ml, both from R&D Systems). Cells were passaged with trypsin (Invitrogen) and DNase I (Roche), and differentiated by removal of the mitogens. For lipofection or differentiation assays, Oli-neu cells were plated at $200x10^3$ cells in 35 mm plates (Corning). For ChIP analysis, cells were expanded in 10cm plates (Corning) and allowed to grow until confluency, before collection.m plates (Corning) and allowed to grow until confluency, before collection.

Primer design. Genbank cDNA sequences or conserved regulatory sequences identified in ECR browser (Loots and Ovcharenko, 2004) were used to design gene specific primers in Primer Express 2.0 (PE Applied Biosystems) or in the Universal ProbeLibrary Assay Design Center (Roche Applied Science). The specificity of PCR primers was determined by BLAST run of the primer sequences. All primers were purchased from MWG Biotech. Primer sequences can be provided upon request.

Immunoblotting. Cells were rinsed twice with cold PBS protease inhibitor (Complete, Roche), collected by scraping and pelleted at 1000 rpm for 2 minutes at 4°C. The cells were then resuspended in NETN buffer (20mM HEPES, 150mM NaCl, 1mM EDTA and 0,5% NP40) with protease inhibitor (Complete, Roche), homogenized by passing through a 21G needle and centrifuged at 13 000 rpms for 15 minutes at 4°C. Supernatant was collected and protein concentration measured. 40µg of protein was mixed with loading buffer containing SDS, glycerol, beta-mercaptoethanol and DTT and run in 12% ReadyGel Tris-HCl (Biorad). After electrophoresis and western blot, cells were probed with the following antibodies: α-HDAC2 (2540), α-HDAC3 (2632), from Santa Cruz Biotechnology, α-Sox10 (AF2864, R&D Systems). After incubation with anti-rabbit and anti-goat HPR antibodies (Chemicon, 1:2000-1:5000), blots were developed using RPN2106 ECL Western Blotting detection reagents, from GE Healthcare.

Reverse Transcription. Total RNA was isolated from NSCs, Oli-neu and CG4 cells using RNeasy extraction kit (Qiagen), with DNAse treatment in-column. 200ng of total RNA were used with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacture's instructions and with each sample being equally divided in two tubes, a cDNA reaction tube and a negative control tube (RT-). Alternatively, Superscript II reverse transcriptase (Invitrogen) was used. Before q-PCR analysis, both cDNA and RT- were then diluted 25 or 50 times, in DNAse and RNAse free dH2O (Invitrogen).

qPCR. qRT-PCR reactions were performed in duplicates for each sample. Each PCR reaction had a final volume of 25 µl and 5µl of 25x- or 50x- diluted cDNA. RT- was run for a few samples in each run to discard genomic DNA amplification. Platinum Quantitative PCR SuperMix-UDG (Invitrogen) was used, according to the manufacture's instructions (but with a 4X dilution from the original mastermix, instead of 2X). The following thermo cycling program was used: 50°C for 2 minutes, 94°C for 2 minutes and then 40 cycles of 94° C for 30 s, 59/60ºC for 30s and 72° C for 30s on the ABI PRISM 7000 Detection System (PE Applied Biosystems, Foster City, CA, USA). A melting curve was obtained for each PCR product after each run, in order to confirm that the SYBR Green signal corresponded to a unique and specific amplicon. Random PCR products were also run in a 2-3% agarose gel to verify the size of the amplicon. Standard curves were generated for every real time PCR run and were obtained by using serial 3-fold dilutions of a sample containing the sequence of interest. Their plots were used to convert Cts (number of PCR cycles needed for a given template to be amplified to an established fluorescence threshold) into arbitrary quantities of initial template for a given sample. The expression levels were then obtained by dividing the quantity by the value of the housekeeping gene, Tata binding protein (TBP). In a few instances, hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as housekeeping gene. For each individual experiment, sample values were normalized by the average sample value of the experiment. TBP assays were run at the beginning and in the middle of assays, to verify the integrity of the samples, and every time the samples were freezed/thawed. Alternatively, the delta-delta Ct method was used.

Immunocytochemistry and cell quantification. Cells were fixed in 10% formalin for 10 minutes and washed in PBS with 0.1% Triton X100, 3 times for 5 minutes. After an overnight incubation at 4ºC with the primary antibody (rat anti-myelin basic protein monoclonal (MAB386, Chemicon, 1:250), mouse RIP (Developmental Studies Hybridoma Bank, 1:100) in PBS with 1-3% bovine serum albumin (BSA), 0.1% Triton X100 and 0,02% sodium azide, the cells were washed six times for 5 minutes with PBS with 0.1% Triton X100. They were then incubated for 2 hours with an appropriate secondary antibody (donkey or goat anti-mouse and anti-rat conjugated to Alexa 488 and Alexa 546/594, 1:500, Molecular Probes/Invitrogen) in PBS with 1% bovine serum albumin (BSA) and 0.1% Triton X100. After 3 washes for 5 minutes with PBS, the cells were either counterstained with 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories). Images were acquired with a Zeiss Axiokop 2 microscope (objectives: 5x/0,25, Zeiss Fluar ∞/0,17, 10x/0,3, Zeiss NeoFluar ∞/0,17 and 20x/0,50, Zeiss Neo-Fluar ∞/0,17) and collected with a Zeiss AxioCam camera MRm (with Axiovison Rel 4.6 software). Images were processed with Adobe Photoshop CS2 version 9.0.2 and panels were assembled with Adobe Illustrator CS2 11.0.1. Quantitative immunocytochemical data of NSCs cultures represents means ± s.e.m., obtained from 3 non-overlapping 10x fields in each condition, from 3 separate independent experiments, after normalization with the number of DAPI positive cells present in the same fields. For DAPI quantification, a macro was programmed in Image J, with the following tools: "Find Edges", "Threshold", and "Analyze Particles". Cells present in clusters were counted manually with the Cell Counter Plug-in. Similar procedures were used in Fig. 4b, in which for each individual experiment, sample values were normalized by the average sample value of the experiment. Oligodendrocyte radius was measured with the AxioVision

Rel 4.6 software. MBP+ cells from 3 non-overlapping 10x field images were counted, in 4 independent experiments. For oli-neu cells, the total number of MBP+ cells with elaborate processes (>3) were counted in the entire 35mm plate in each independent experiment and then normalized by the number of DAPI+ cells in 5 random fields. For each individual experiment, sample values were normalized by the average sample value of the experiment.

RNA profiling. RNA was prepared from cultured cells of E12.5 cortex from wild-type NSCs 3 h after plating. RNA quality was assessed using the Agilent Bioanalyser 6000 Pico LabChip. 100 ng of total RNA was labelled with Cy-3 or Cy-5 using the Agilent Low RNA Input Fluorescent Linear Amplification Kit. A dye-swap design was employed. Labeled cDNA was hybridized to the Agilent 44K Whole Mouse Genome Array. Data was collected using the Agilent Microarray Scanner and Feature Extraction Software, using a Lowess option with spatial detrend to extract genes of interest with more confidence than through the use of fold-change only. Experiments were performed in triplicate, with litter-matched wild-type and mutant samples.

ChIP-seq in cortically-derived embryonic NSCs. Chromatin immunoprecipitation (ChIP) was performed following the High Cell ChIP kit # protocol from Diagenode. Five micrograms of anti-HDAC antibodies were used in each IP. Initial ChIP analysis was done with qPCR using Invitrogen Platinum SYBR Green qPCR Supermix-UDG together with site-specific primers. Chromatin immunoprecipitation followed by next generation sequencing (ChIP-Seq) was performed essentially as described before (Füllgrabe et al, 2013; Heldring et al., 2014). For ChIP-seq analysis, 5 mg of

chromatin was used in 2 separate IP's and combined into one elution. Subsequently, the DNA sequencing library was made using a kit from Illumina, except Illumina TruSeq adaptors were used to enable multiplexing. The library was analyzed by Solexa/Illumina Hi-seq. After prefiltering the raw data by removing sequenced adapters and low-quality reads, the sequence tags were aligned to the human genome (assembly hg19) with a Bowtie alignment tool (Langmead et al., 2009). To avoid any PCR-generated spikes, we allowed only one read per chromosomal position and thus eliminated PCR bias. From the filtered raw data, 2 million unique reads per sample were used for peak detection. Peak detection was per- formed using the CisGenome program (Ji et al., 2008) with a 2-sample analysis where sequenced input (1%) was used as a negative control. Peaks were called with a window statistic cutoff of 3 and a log2 fold change of 2.

REFERENCES

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Heldring, N., Nyman, U., Lönnerberg, P., Önnestam, S., Herland, A., Holmberg, J., and Hermanson, O. (2014). NCoR controls glioblastoma tumor cell characteristics. Neuro-Oncol. *16*, 241-249.

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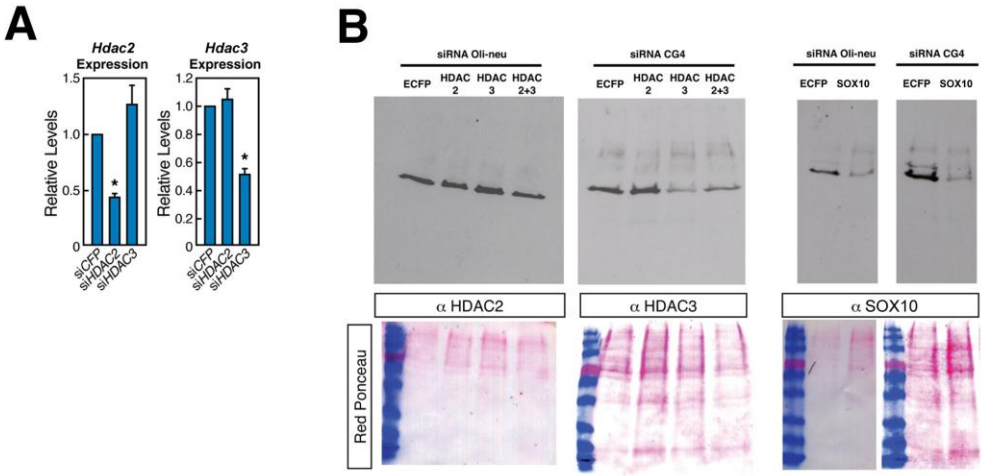
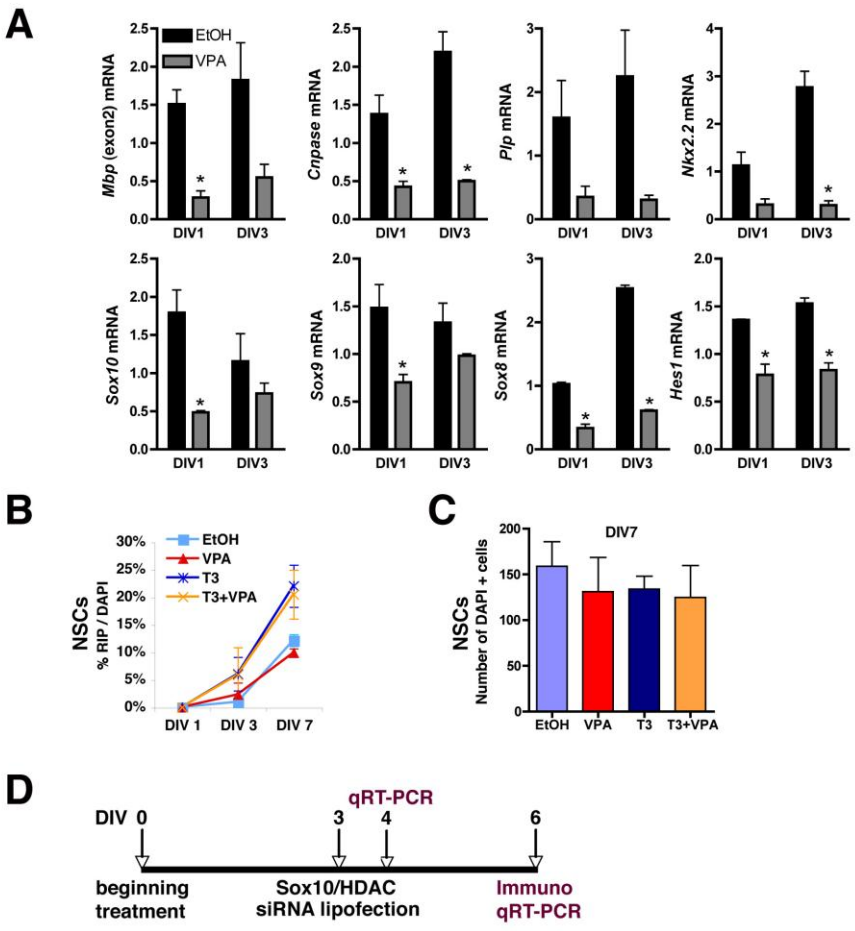
Loots, G.G., and Ovcharenko, I. (2004). rVISTA 2.0: evolutionary analysis of transcription factor binding sites. Nucleic Acids Res. *32*, W217-221.



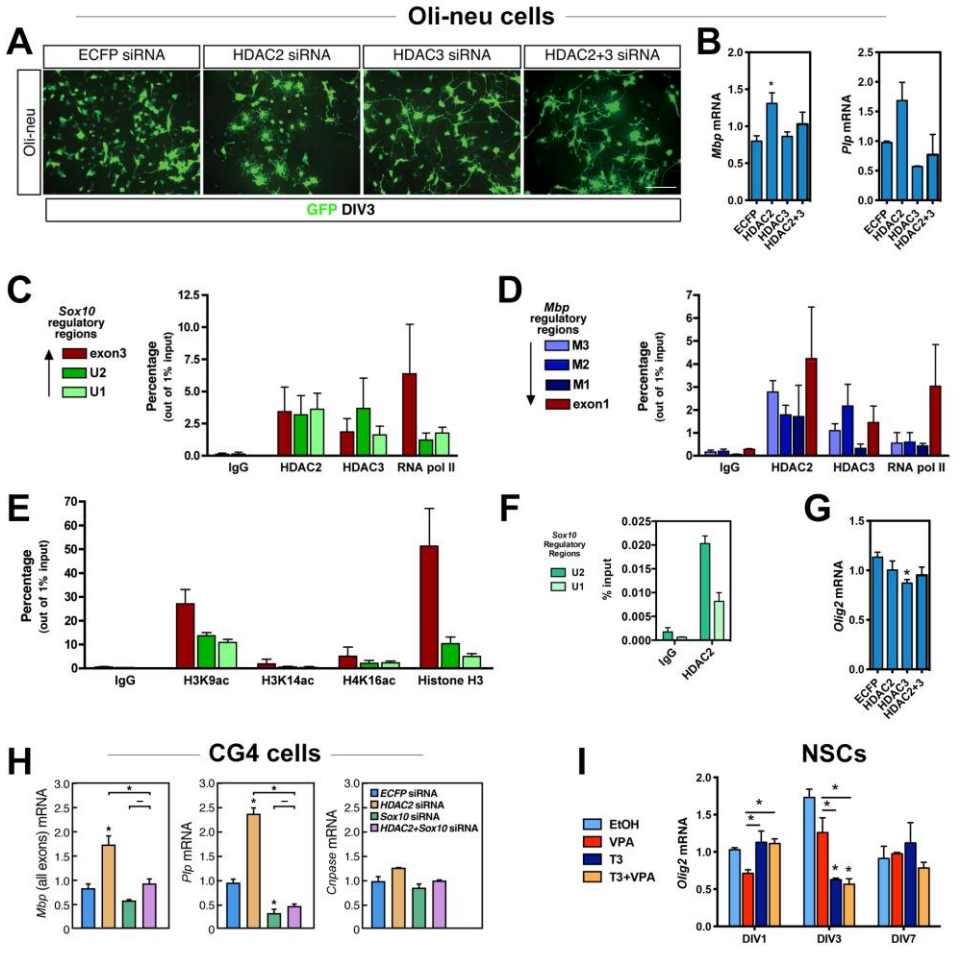
Supplementary Figure S1

A. Bars representing the levels of *Hdac2* (left panel) and *Hdac3* (right panel) mRNA as assessed by qRT-PCR after siRNA against *Hdac2* (siHDAC2) and *Hdac3* (siHDAC3).
B. Lipofection of *Hdac2* , *Hdac3* and *Sox10* siRNAs in Oli-neu and CG4 cells led to a specific decrease in the levels of the respective proteins,1 DIV after lipofection, as assessed by immunoblot with antibodies against HDAC2, HDAC3 and SOX10. Equal amounts of protein were loaded.
A-B: n=3-5 independent experiments. Error bars = S.E.M..



Supplementary Figure S2

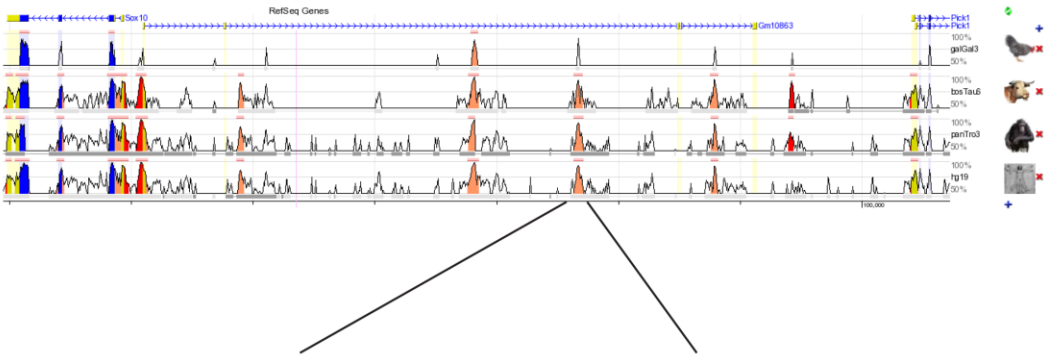
A. qRT-PCR analysis of oligodendrocyte-associated transcripts revealed that HDAC inhibitor VPA in neural stem cell cultures treated with bFGF, PDGF, and IGF-2 (OLPm) led to decreased transcription of *MBP*, *CNPase*, *Nkx2.2*, *Sox10*, *Sox9*, *Sox8*, and *Hes1*.n=3-5 independent experiments.
*p<0.05 (t-test), error bars - standard error of the mean (s.e.m.).
B. The percentage of immature oligodendrocytes (RIP+) out of the total cell population (DAPI+) increased upon treatment with T3, but was not altered by VPA, as assessed by immunocytochemistry.
C. The total number of cells was not significantly affected by any of the treatments, as assessed by quantification of the number of DAPI+ cells. (B-C, n=3 independent experiments.)
D. Scheme of *Sox10/Hdac2/3* knockdown in differentiating NSCs. NSCs were differentiated for 3 DIV in absence of FGF-2 and in the presence of the different compounds. At DIV3, NSCs were lipofected with *Sox10*/*Hdac2*/*Hdac3* siRNA. Immunocytochemistry and qRT-PCR were performed with samples collected at DIV4 (1 DIV after lipofection = 1 DAT) and DIV 6 (3 DIV after lipofection = 3 DAT).



Supplementary Figure S3

A. Proliferating prenatal oligodendrocyte progenitors (Oli-neu cells) were co-lipofected with HDAC2 and/or HDAC3 siRNAs and a plasmid containing green fluorescent protein (GFP). Both siRNAs induced clear morphological changes with HDAC2 siRNA resulting in multiple short processes and HDAC3 siRNA rather to the appearance of thin, long processes. Scale bar: 60µm.
B. RT-qPCR results demonstrating *Mbp* and *Plp* mRNA levels in differentiating Oli-neu cells that were lipofected with control, HDAC2 and/or HDAC3 siRNA.
C, D, E, F. Chromatin from proliferating Oli-neu cells was cross-linked with 1% formaldehyde for 10 minutes as described in Experimental Procedures, and ChIPs were performed using antibodies against HDAC2, HDAC3, PolII, Histone H3 and the acetylated lysines H3K9, H3K14 and H4K16, followed by qPCR of regions on defined regions on the *Mbp* and *Sox10* loci.
G. RT-qPCR results demonstrating *Olig2* mRNA levels in proliferating Oli-neu cells that were lipofected with control, HDAC2 and/or HDAC3 siRNA. No increase in expression was observed.
H. RT-qPCR results demonstrating *Mbp*, *Plp*, and *Cnpase* mRNA levels in postnatal oligodendrocyte precursor (CG4) cells that were lipofected with control, HDAC2 and/or Sox10 siRNA.
I. RT-qPCR results demonstrating *Olig2* mRNA levels in NSCs that had been treated with vehicle, VPA and/or T3. No increase in expression was observed.
A, B, G, H, I: n=3-5 independent experiments. **C, D, E, F:** n=2-3 independent experiments. Error bars = S.E.M..

A



Sox10 **U2 enhancer sequence in mouse (mm10)/human (hg19)**

Conserved SOX_Q6 binding sites in blue (analysis with ECR browser and RVISTA 2.0)
ChIP-qPCR U2 amplicon in red

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mm10    CACTTCTCTCAGCCTCAGTTTCCCTCTCTTGTAATACAGGGGTGAATTGTCTCCATAACC
hg19    CATCTCTCTGGGTCTCAGTTTCCCCCTCT-ATAACACCAGGG--GCTGGTCTTCATGATC

mm10    GCAAGACCCTCTCCAGCTGTGCCCTCCTGTGACTGAGAG--TCTGAGAGCAGAGGCAGGA
hg19    CCAGGGCCCTCCCCAGCTGTGCCCATCTGCAACTGTGAGATTCTGAGAGCAGAGGAGGGA

mm10    AGGCAAGATGGGGCCCCTCTTTCCCCACCTCCTCTCCCTGTCCCCCAGGGTCACCCCAAA
hg19    AGGCGGGATGG--CCCCTCTTTCCCCACCGCCTCTCTCTGTCCCCCAGGGTCACCCCAAA

mm10    GCCGCCCCT--CCCCAGCTCCCTGGGACTTCCCTGCAGCTGCAGAC-TGGGGAGACAATC
hg19    GCCGCCCCCTCCCCCGGCTCCCTGGAACTTCCCTGCAGCTGCAGGCCTGGTGAGACAATT

mm10    GGGTGCTTTAGAGAAATACATTTTGGGCTAATTTGCCATGCAGAAGTGTTTCTCTTACC
hg19    GGGTGTTTTAGAGAAATACATTTTTGGGCTAATTTGCCATGCATAAGTGTTTCTCTTACC

mm10    CTTTAAAGGCAGAGAACAAAGAGACCTTTCTGTGCCTGGGCAGGCTGCTGGGAAGGGGAC
hg19    CTTTAAAGGCGGAGAACAAAGAGGTCTTTCTGTGCCCAGGCAGGCTGCTGGGGAGGGGA-

mm10    CGCAGCTGTA-GGGGCTGTCGAC---CCTCCCTCCCTCCATCCCAGTGAGCATCCCAGCA
hg19    CGCAGCTGTACTGGGCTGGCGACCCTCCTCCCTCCCCCCATCCCTGCGAGCGCCCCACCA

mm10    CACAGCCCCCGTGTCCCACCCCCGCCCCCAAGAGGGACAGCCTGGACTTTCCCAGCCTC
hg19    CACCG---------CCCACCGC---CCCCCAGAAGAGACAGCCCGGACTTTCCCAGCCTC

mm10    CCCCAGTTGCCCAGCCGGG----------------------------------CCCAGCCCC
hg19    CCCCAGTCTCCTACCCCGGCTGCCTCTGAGGCTCCTCTGGCCCTGCCTTGCCCCAGCTCC

mm10    CTCCCACCAGCTGCCCCAGGCGAGAGGGAAAGTGTGCGTGGGT-GGCGACTGGGTGGGGA
hg19    CCCTC-CCAGCTGCCCCAGGCAAGAGGGAAAGTGTGTGTGGGTGGGTGGATGGACGGGGA

mm10    GGGGC--ACCAGGAGAGAAAGCCATTCTCCACGTGGTTGTGGTG
hg19    GGGGCGTGCATGGAGAGACAGCGGCTTTCCATGTGGCCCGGGTG
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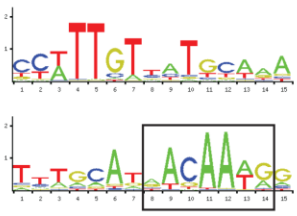
B

Enriched binding motif for SOX2
(source JASPAR database, ID MA0143.1,
derived from Chen et al., Cell 2008)



Reverse complement

Supplementary Figure S4

A. A conserved SOX2 binding site (blue) in the U2 enhancer (red) of the *Sox10* gene found in mouse and human.

B. A similar site detected by ChiP-Seq in embryonic stem (ES) cells.

Reference:
Chen et al. (2008) "Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. Cell *133*, 1106-17.

CASTELO-BRANCO et al, SUPPLEMENTARY TABLE 1.
Gene expression data underlying heatmap in Figure 2B.

PEAK FOLD	ACC #	DESCRIPTION
25.14	M37335	proteolipid protein (myelin)
28.74	M11533	myelin basic protein
7.47	M63801	gap junction membrane channel protein alpha 1
4.84	X16202	
4.59	V00746	histocompability 2, K region
4.41	M69069	histocompability 2, D region locus 1
4.19	AI117211	histocompability 2, L region
3.79	C78850	RIKEN cDNA 1300007C21 gene
3.79	L26836	ATP-binding cassette, sub-family D (ALD), member 3
4.18	L47480	bone morphogenetic protein 4
5.14	M28730	tubulin, beta 4
4.00	M16472	proteolipid protein (myelin)
4.04	AF031127	
3.75	K02236	metallothionein 2
3.39	U31566	NK2 transcription factor related, locus 2 (Drosophila)
3.12	AJ223206	scrapie responsive gene 1
3.16	M26071	coagulation factor III
3.52	X52490	histocompability 2, D region
2.79	M25944	carbonic anhydrase 2
2.78	X01838	beta-2 microglobulin
2.77	X00246	histocompability 2, D region locus 1
2.72	AJ006474	carbonic anhydrase 3
2.95	AI847230	
2.97	AI851348	
2.73	U73521	solute carrier family 1, member 1
2.60	AI840267	sirtuin 2 (silent mating type information regulation 2, homolog) 2 (S. cerevisiae)
2.69	J04627	methylenetetrahydrofolate dehydrogenase (NAD+ dependent)
2.35	M21265	stearoyl-Coenzyme A desaturase 1
2.24	J02652	malic enzyme, supernatant
2.42	L06115	CD9 antigen
2.58	M18837	
2.43	M58156	MHC (A.CA/J(H-2K-f) class I antigen
2.44	AI850558	
2.17	M27134	histocompability 2, K region locus 2
2.98	AW060549	RIKEN cDNA 13000007C21 gene
2.67	AF017994	mesoderm specific transcript
2.56	M17327	
2.69	AI845796	RIKEN cDNA 231000B05 gene
2.22	AI842277	insulin-like growth factor binding 3.
5.20	U19582	claudin 11
3.70	M63801	gap junction membran channel protein alpha 1
3.57	L22144	S100 protein, beta polypeptide, neural
3.44	AB017270	transmembrane protein with EGF-like and two follistatin-like domains 2
3.45	AW046181	serum/glucocorticoid regulated kinase
3.31	U51000	distal-less homeobox 1
3.05	AI845514	ATP-binding cassette, sub-family A (ABC1), member 1
2.66	AW125478	protease, serine, 11 (igf binding)
2.67	AI840191	expressed sequence AW547365
2.28	D83277	RAB33A, member of RAS oncogene family

2.23	AI001972	inhibitor of DNA binding 4
2.21	AW124983	epidermal growth factor receptor pathway substrate 15
2.19	L12447	insulin-like growth factor binding protein 5
2.20	AB017026	cDNA sequence AB017026
2.18	AI842472	
2.23	AI848201	RIKEN cDNA 1700006H23 gene
2.33	AF031127	
2.17	AW124196	RIKEN cDNA 5530600A 18 gene
2.21	X66449	S100 calcium binding protein A6 (calcyclin)
2.24	AI847795	
2.24	U86090	kinesin family member 5B
2.39	U39738	p21 (CDKN1A)-activated kinase 3
2.53	AW122114	RIKEN cDNA C0300448H19 gene
2.58	AW061337	adenylate kinase 4
2.27	L49507	cyclin G
2.22	AW125874	RIKEN CDNA 3010001M15 gene
2.23	AW046627	
2.16	AI842065	expressed sequence AU046135
2.23	AW125390	RIKEN cDNA 1110004C05 gene
2.26	AA016517	RIKEN cDNA 1500005102 gene
2.15	AI844626	glycine amidinotransferases (L-arginine glycine aminotransferas)
2.30	AV347220	endothelial differentiation sphingolipid G-protein-coupled receptor 1
2.35	X05862	
-2.81	AI851048	RIKEN cDNA 1810030E20 gene
-2.60	AI854154	DNA segment, Chr 9, Wayne State University 18, expressed
-2.64	AI853439	
-2.90	X61397	carbonic anhydrase-like sequence 1
-4.24	AA880275	metallothionein-I activator
-6.72	AA874329	
-3.67	L04961	inactive X specific transcripts
-7.34	AW047207	RIKEN cDNA 1810037I17 gene
-4.75	AI853444	RIKEN cDNA 2610042L04 gene
-3.77	AI853444	RIKEN cDNA 2610042L04 gene
-6.33	AI854020	cysteine dioxygenase 1, cytosolic
-4.03	AF084642	retinaldehyde binding protein 1
-2.94	AF000294	peroxisome proliferator activated receptor binding protein
-4.07	AW228316	RIKEN cDNA 2310046G15 gene
-3.23	AB028241	casein kinase 1, epsilon
-2.74	AW258842	RIKEN cDNA 2510049I19 gene
-2.27	U32329	endothelin receptor type B
-2.43	AI325791	expressed sequence AI507524
-2.36	U22399	cyclin-dependent kinase inhibitor 1C (p57)
-2.73	X15986	lectin, galactose binding, soluble 1