Supplemental Data
Histone Methylation-Dependent Mechanisms Impose Ligand Dependency for Gene Activation by Nuclear Receptors

Ivan Garcia-Bassets, Young-Soo Kwon, Francesca Telese, Gratien G. Prefontaine, Kasey R. Hutt, Christine S. Cheng, Bong-Gun Ju, Kenneth A. Ohgi, Jianxun Wang, Laure Escoubet-Lozach, David W. Rose, Christopher K. Glass, Xiang-Dong Fu, and Michael G. Rosenfeld

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Materials and Reagents

The following commercial available antibodies were used: α-ERα, CBP, and Pol II from Santa Cruz Biotechnology, α-dimethylH3K4, acH3K9, and ESET from Upstate Biotechnology, α-BHC110 from Bethyl Laboratories, α-dimeH3-K9 and RIZ1 from Abcam, and α-RIZ1 from Orbigen. 17-ß-Estradiol (E_2), 4-hydroxytamoxifen (4-OHT), and Dihydrotestosterone (DHT) were purchased from Sigma. TNFα and RA were from R&D Systems. Human IL-1ß from Calbiochem. The concentrations of treatment were: E_2 (100 nM), 4-OHT (1 μM), DHT (20 nM), RA (1μM), IL-1ß (10 ng/ml), TNFα (10 ng/ml). The specific and non-specific control siRNAs were purchased from Qiagen.

ChIP-DSL analysis

The ChIP-DSL assays were carried out as described in Kwon et al., 2007. Briefly, the ChIP-DSL technology utilizes a 40mer array for hybridization. Each 40mer corresponds to a unique genomic sequence identified by computational search. For promoter profiling, each such probe corresponds to a proximal promoter region from +200 nt to -800 nt relative to the transcriptional start site. For tiling profiling, each probe represents a 0.5 kb non-repetitive

genomic block in a path to be tiled. Amine-modified 40mers are spotted onto solid support to form an array.

The assay begins with the standard ChIP procedure. About $1x10^6$ cells were used for each ChIP-DSL experiments. Both input (5% of total DNA) and antibody-enriched DNA were isolated and randomly biotinylated. Then annealed independently to a mixture of 20,045 oligonucleotide pairs, each of which targets to a specific 40mer promoter sequence in the human genome. Each oligonucleotide consists of one half of the corresponding 40mer sequence, flanked by a universal primer-landing site (T3 for the upstream and T7 for the downstream oligonucleotides). After annealing, streptavidin beads are used to select annealed oligonucleotides templated by input or ChIPed DNA and later, free oligonucleotides are removed by sequential washes. Then, precisely paired oligonucleotides are ligated on beads by the Taq ligase. Through this DNA Selection and Ligation (DSL) step, only correctly targeted oligonucleotide pairs are converted from half to complete amplicons, which are PCR-amplified by using the universal T3 and T7 primers. One of the primers is end-labeled with Alexa dye (for total genomic control) or Cy3 dye (for ChIPed DNA), and the PCR products are co-hybridized to the 40mer array (Hu20K or tiling array) to derive enrichment ratio for each target. For data analysis, the single error model was applied as previously described (Li et al. 2003; Ren and Dynlacht, 2004).

Single-Cell Nuclear Microinjection Assays and *LacZ* Reporter Assays

Microinjection assays were carried out as previously described (Kamei et al.,1996; Jepsen et al., 2000). Briefly, each experiment was performed on three independent cover slips consisting of 1,000 cells, with >300 cells injected. Where no experimental siRNA was used,

unspecific or *GST* control siRNA was microinjected. Injected cells were distinguished from un-injected cells by the co-injection of an inert dextran that is fluorescently labeled. Approximately 1 hr after injection, the cells were treated, where indicated, with ligands/reagents. After overnight expression, the cells were fixed with 3.7% formaldehyde and then subjected to X-gal staining. Results were quantitated after X-gal staining, and each fluorescent injected cell was individually scored as positive or negative for *LacZ* expression.

Quantitative ChIP-PCR (ChIP/qPCR)

Specific PCR primers designed surrounding a region of 150-250 bp from the DSL probe were used to determine enrichment of specific sequences by ChIP assay. This enrichment was determined by quantitative real time PCR and SYBR Green-detection (Stratagene) on a Mcx300P System (Stratagene). PCR reactions typically used 1 μL of sample from 60 μL DNA extraction. Length of PCR products was confirmed by agarose gel. Fold enrichment ratios were calculated from experimental Ct values, previously normalized against Ct values from IgG control, and then input percentages were calculated compared to serial diluted input samples.

ChIP/qPCR PRIMERS LIST				
PROMOTER	**FORWARD PRIMER**	**REVERSE PRIMER**	**Band Size**	**Verification on agarose gel**
ABP1/DAO	GAAATGGCCCCTTGTCCT	GCCTATGGCTTGGGAGGT	219 bp	OK
DSCR3	GAGGGATTGTGGGTGTTCC	GGCAGATTCACTCCAGCTTT	158 bp	OK
BLVRB/SPTBN4	GCCACGCCTCAGACTCTC	GGGCGCTGAGTGACAAGT	211 bp	OK
C11ORF30/EMSY	GGGTATGCAGGAGAAACCAA	AGATGGCGACAGGTAACAGG	274 bp	OK
C21ORF13	GCCTGGAGGTCAGTGTGG	GTGACAGGCTCCCTCGTCT	152 bp	OK
CBX5/HNRPA1	TCTCAGAGGCAGGTGGAACT	TTCCACGCGCAAATATTACA	173 bp	OK
CCND1	GCCGGCCTTCCTAGTTGT	GCCCCTGTAGTCCGGTTT	167 bp	OK
CRKL	CAGTCCTTGACCTCCCTCAG	GGCAGGAAAACAGCTGGAG	151 bp	OK
DDX17	AAGTACGGAGCGAGCGTAGA	GAGATCGGAATCAGGCAAAA	184 bp	OK
ESR1	GTCCTCCCCAGGGTCATC	CCAGGGAAGACTGGGCTTA	186 bp	OK
GAPD	TCCTCCTGTTTCATCCAAGC	TAGTAGCCGGGCCCTACTTT	219 bp	OK
GMIP	CCTGACCTGGGCATCCTA	AGGTGGTTTCAGGGCTCC	209 bp	OK

GREB1C	TTGTTGTAGCTCTGGGAGCA	CAACCAGCCAAGAGGCTAAG	249 bp	OK
GREB1C distal	GAGCTGACCTTGTGGTAGGC	GGTTTTTAAGCAGCCAGCAG	165 bp	OK
C2orf13	GCCTTTGGGGATTGGTAGTT	CCCTCCACGCCTACTAATGA	205 bp	OK
HIG2	ACGCCTCGTCTCACTCCA	CCTTATAGCCGCGAGCAC	214 bp	OK
LOC55954/ZMAT5	GCTTGCTGGGATTCGTAGTC	TCTCCAGAATTCCTGGCTGT	164 bp	OK
MAP2K7	CCACCGTCCTCAGAAGAATG	GAAGGATGACGCCACCTAGA	145 bp	OK
MAX	CCCCACACACACACTCACTC	GCCGTCTGTTAGAGGGACAA	194 bp	OK
NR1D1	CGACAAGACTGTCGGGATTT	CTAAACACGCCACCCTGACT	152 bp	OK
C11orf4	CGGACCCCCATACAAGTAAA	GCGTGAAACCGTACGAGAAT	164 bp	OK
BANP	CGGAGTCCGGAGAGACTACA	CAGTGGACGGTCTGAGGAAT	176 bp	OK
OVGP1	CCCAGTGCCAGGATCAGT	TGGGGTTTCTCCCCCATA	161 bp	OK
PHB	CCTGCTTCCACTCTGACCTC	TGCATTTAGCCCCAGAAAAC	299 bp	OK
PPP1R15A	TCCGCGAGAAGTCCTGTC	GTATCTCCGGCTGCCTCA	228 bp	OK
PSCD2	GCGGAGAAGAAATGCAAAAG	GGGGGCGGTACTTTGTTAGT	163 bp	OK
RANBP3	CAGTGGCAGCGACTAAGGTT	GCTCCTAAGGCGGGTAAACT	231 bp	OK
RPS6KB1/TUBD1	ACAAATGCGCATGTTCCATA	GTTCCGTGAAGCCAAACAGT	270 bp	OK
SMARCA4	ATGGCACTTGGGAAGTTTTG	GAGGCTCGGAAATAGGAACC	191 bp	OK
SYN1	TCTGAGTGTGCTTCCAGTGC	GATAGGGGATGCGCAATTT	215 bp	OK
RNF14	TTTGTGAGGATTTCCCAAGC	GCAACCCTACCTCAGAGTGC	104 bp	OK
TFF1/pS2	GCTTAGGCCTAGACGGAATGGGC	CCAGGTCCTACTCATATCTGAGAG	189 bp	OK
TFF1/pS2 distal	CTGGGTGACAGGAAAGAAGC	CATTCTGGAAGGGACACACA	244 bp	OK

Quantitative RT-PCR

Total RNA was prepared from samples by the RNeasy® Mini Kit (Qiagen). One μg RNA was treated with DNase I (Invitrogen) to remove any trace of DNA. Quantitative PCR was performed by using SYBR Green (Stratagene) on a Mcx300P System (Stratagene). The relative mRNA level was calculated by comparing with non-treatment or control, after normalization with *ACTB* mRNA levels.

qRT-PCR PRIMERS LIST				
PROMOTER	**FORWARD PRIMER**	**REVERSE PRIMER**	**Band Size**	**Verification on agarose gel**
GREB1	GGCAGGACCAGCTTCTGA	CTGTTCCCACCACCTTGG	237bp	OK
MYC	CCTACCCTCTCAACGACAGC	CTCTGACCTTTTGCCAGGAG	239 bp	OK
pS2/TFF1	TTGTGGTTTTCCTGGTGTCA	CCGAGCTCTGGGACTAATCA	209bp	OK
CTSD	GGTGGCACAGACTCCAAGTA	ATGAGGGAAGTGCCTGTGTC	162 bp	OK
WISP2	CTGTATCGGGAAGGGGAGAC	GGGAAGAGACAAGGCCAGAA	247bp	OK
ACTB	GTGGGCATGGGTCAGAAG	TCCATCACGATGCCAGTG	329bp	OK

Clustering analysis

Binding profiles of di-methyl H3-K4, acetyl H3-K9, Pol II, and LSD1 were represented as 1 for binding and 0 for no binding. Expression profiles at 6 and 12 hr time points were represented as 1 for expression and 0 for no expression. All binary profiles were clustered using Pearson correlation and average-linkage hierarchical clustering (Eisen et al., 1998). Gene expression profiles of 79 normal human tissues were obtained from the Gene Expression Atlas available at http://symatlas.gnf.org (Su et al., 2004). Relative expression ratios were obtained by dividing each value by the row-wise average. Expression ratios were log transformed and median centered by genes and arrays. Unsupervised hierarchical clustering was performed using average-linkage clustering.

Supplemental References

Eisen, M.B., Spellman, P.T., Brown, P.O., and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. U.S.A. *95*, 14863-14868.

Jepsen, K., Hermanson, O., Onami, T.M., Gleiberman, A.S., Lunyak, V., McEvilly, R.J., Kurokawa, R., Kumar, V., Liu, F., Seto, E., et al. (2000). Combinatorial roles of the nuclear receptor corepressor in transcription and development. Cell. *102*, 753-763.

Li, Z., Van Calcar, S., Ou, C., Cavenee, W.K., Zhang, M.O., and Ren, B. (2003). A global transcriptional regulatory role for c-Myc in Burkitt's lymphoma cells. Proc. Natl. Acad. Sci. U.S.A. 100: 8164-8169.

Ren, B. and Dynlacht, B.D. (2004). Use of chromatin immunoprecipitation assays in genome-wide location analysis of mammalian transcription factors. Methods Enzymol. 376: 304-315.

Su, A.I., Wiltshire, T., Batalov, S., Lapp, H., Ching, K.A., Block, D., Zhang, J., Soden, R., Hayakawa, M., Kreiman, G., et al., (2004). A gene atlas of the mouse and human proteinencoding transcriptomes. Proc. Natl. Acad. Sci. U.S.A. *101*, 6062-6067.

Figure S1. Test on efficiency and specificity of the guinea pig α-LSD1 antibody generated. **(A)** Western blot analysis with the a-LSD1 antibody generated against bacterially-expressed LSD1 in MCF7 whole cell extract. **(B)** Immunostaining of overexpressed FLAG-LSD1 in 293 cells using α-LSD1 and α-Flag antibodies. DAPI staining control (left) and α-LSD1/α-Flag merged images (right) are shown. **(C)** Immunoprecipitation of LSD1 and coimmunoprecipitation of CoREST from MCF7 cellular extracts by using α-LSD1 antibody. Actin control is also shown. **(D)** ChIP with a-LSD1 antibody performed on previously-reported LSD1 genomic targets (Shi et al. 2004).

Figure S2. Genomic localization of DSL probes relative to reported LSD1 binding sites. **(A)** Only two previously-reported LSD1 genomic target sites were located <50bp from a DSL probe represented on the Hu20K array and their regulated gene showed similar expression status in MCF7 cells (top box): the repressed *SYN1* and *SCN3A* promoters; and both were $LSD1^+$ by ChIP-DSL. Another reported LSD1 target, the repressed *p57*/*CDKN1C*, was $LSD1^-$ (middle box); but the DSL probe was located >190bp from the PCR-amplified product reported by ChIP. Three other reported LSD1 targets: *SCN2A2*, *CHRM4*, and *SCN1A*, were located >1.5 Kbp from the DSL probe (middle box), thus indeed not being represented on the Hu20K array. Finally, two additionally genomic sites, located <50bp from the reported LSD1 binding site, showed a completely different expression status in MCF7 cells than when reported LSD1 targets: *PSA*, a prostate-specific gene, expressed in LNCaP, but not in MCF7 cells; and *FOS*, an expressed and E2-induced gene in MCF7 cells, but repressed when previously reported LSD1-target in 293 cells (bottom box). **(B)** BLAT search analysis showing

the genomic distances between reported LSD1-enriched genomic sites and DSL probes (UCSC Browser, http://genome.ucsc.edu/).

Figure S3. Multiple neuronal NRSF/REST-target genes repressed in non-neuronal cells were detected as LSD1$^+$. (A) Partial list of literature-reported or computational-predicted NRSF/REST target genes within the group of LSD1$^+$. **(B)** Average linkage hierarchical clustering of RNA transcript levels (upper panel) of 79 normal human tissues (bottom list) obtained from the Novartis v2 expression data set (GNF: http://symatlas.gnf.org) showing a cluster of neuronal-specific genes (II). Non-expressed genes in MCF7 cells from this cluster (data not shown) were classified based on their LSD1 recruitment status at P<0.0001 (left column): 94 LSD1$^+$ and 529 LSD1$^-$ genes. Motif analysis on the promoters of these genes showed a significant enrichment of NRSE/REST sites on the group of neuronal LSD1$^+$ genes (6.4% RE1-containing promoters) compared to a random selection of promoters (0.4% RE1-containing promoters). A significant enrichment was also observed for the group of neuronal LSD1- promoters (3.9% RE1-containing promoters), but this enrichment was lower, thus confirming an association between LSD1 binding and NRSF/REST enrichment.

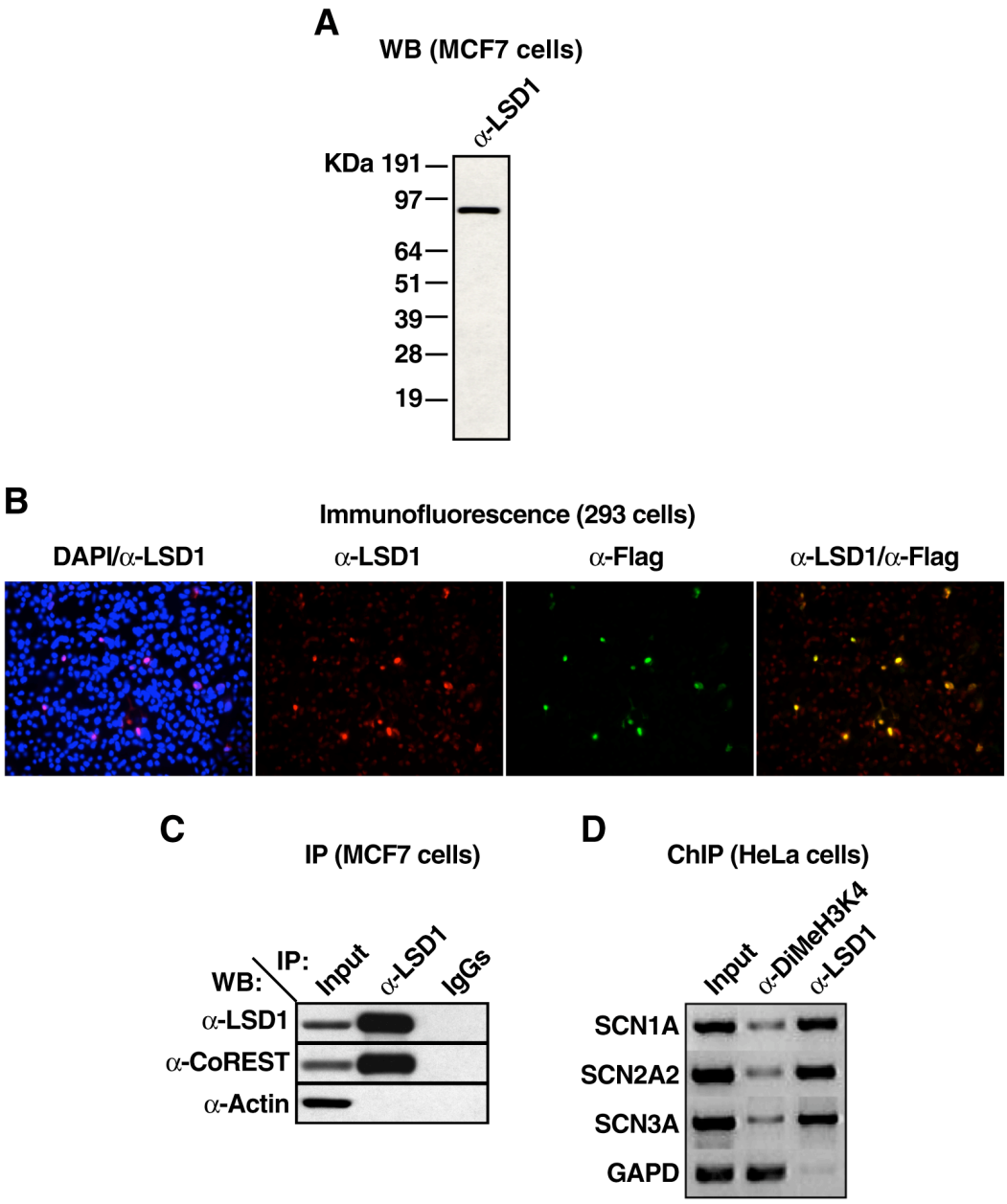
Figure S4. Knock down efficiency of specific siRNAs determined by Western blot analysis. (A-B) Protein levels of LSD1 (A) and ESET (B) determined by Western blot using antibodies specific for the depleted proteins upon siRNA transfection. Protein levels of ERα were examined in cells treated with *LSD1* siRNA.

Figure S5. LSD1-dependent regulation of an ERE-Luciferase reporter gene. Luciferase assay using a multimerized ERE-*Luciferase* reporter gene vector to report ERα-dependent activation in cells overexpressing a wild-type or a mutant form of LSD1. Numbers refer to amounts of expression plasmid (ng). The data are the average of three replicates, and error bars represent ± standard error mean.

Figure S6. LSD1 regulates other induced transcriptional programs. (A). Effect of *LSD1* siRNA on transcription units previously reported to be regulated by LSD1. The *PSA-LacZ* reporter activity is measured in LNCaP cells stimulated with DHT (left) and a multimerized RE1-LacZ reporter activity is measured in Hela cells (right) upon siRNA microinjection. **(B)** Additional LSD1 regulated transcription units. The activity of *LacZ* reporters controlled by AP-1 (left), RARE (middle) or NF B (right) sites are measured in cells microinjected with a *LSD1* siRNA and stimulated by corresponding ligands (TPA, RA and TNFα). **(C)** The effect of LSD1 depletion by siRNA on the activity of a *LacZ* reporter controlled by a CRE site upon forskolin treatment. **(D)** The effect of LSD1 depletion by siRNA on the activity of a *LacZ* reporter controlled by the *pS2* promoter upon E2 treatment. For all experiments, reporter plasmid and *LSD1* siRNA were delivered by single-cell nuclear microinjection in HeLa, LNCaP (in A), and MCF7 (in D) cells. For each reporter assay the nuclear receptor or transcription factor implicated has indicated. The data are the average of three replicates, and error bars represent ± standard error mean.

Figure S7. Ligand/signal-dependency imposed by the opposing functions of H3-K9 HMTs and LSD1 on distinct transcriptional programs. (A- B) A *LacZ* reporter activity regulated

by the proximal *PSA* (A) or *E-selectin* (B) promoters was analyzed in cells treated with specific *H3-K9 HMTs* siRNAs, separately or together with *LSD1* siRNA in absence of ligand (DHT or TNFa). **(C)** RT-qPCR analysis of an endogenous NF B-target gene induced by TNFα treatment upon *RIZ1/ESET* and/or *LSD1* siRNA transfection. **(D)** Functional *E-Selectin* promoter-*LacZ* reporter gene analysis of NFκß (p50/p65 factors)-dependency in absence of signal (TNFα) and specific H3-K9 HMTs. In A, B, and D, reporter plasmid and siRNAs were delivered by single-cell nuclear microinjection in HeLa cells. In C, siRNAs was delivered by transient transfection in HeLa cells. The data are the average of three replicates, and error bars represent ± standard error mean.



Garcia-Bassets et al., 2006 Figure S1

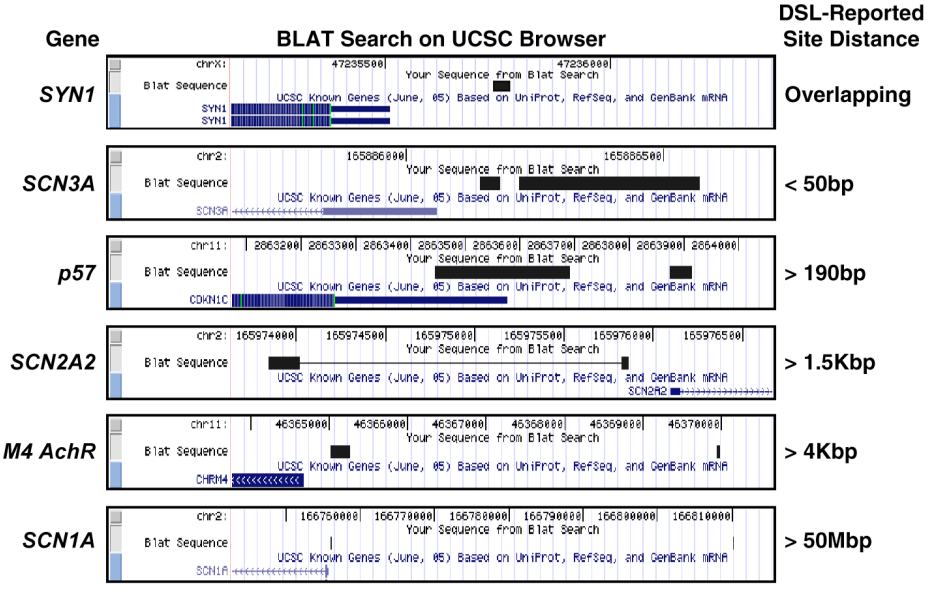
A

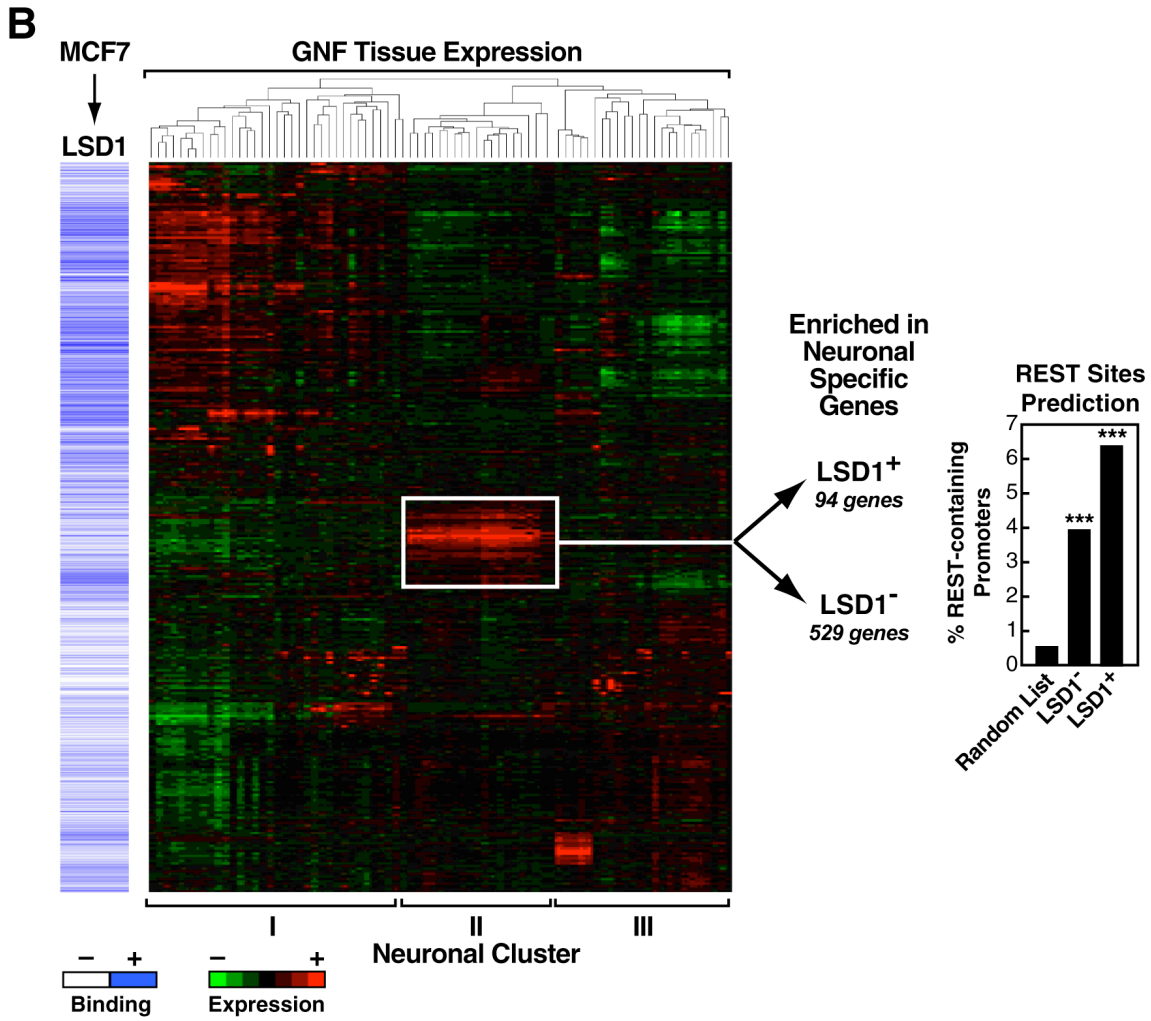
Situation of the Reported LSD1-Regulated Target Genes in the ChIP-DSL Experiment

Target Gene	DSL Probe & Reported LSD1 Binding Site Distances	Gene Status when LSD1 Bound	Gene Status in MCF7 Cells	ChIP DSL Results	Reference
Reported LSD1 Target Sites Represented on the Hu20K Array					
SYN1	Overlapping	Repressed	Repressed	LSD1$^+$	Hakimi *et al.*, 2002
SCN3A	< 50 bp	Repressed	Repressed	LSD1$^+$	Shi *et al.*, 2004
Reported LSD1 Target Sites Non-represented on the Array					
p57/CDKN1C	> 190 bp	Repressed	Repressed	LSD1$^-$	Shi *et al.*, 2004
SCN2A2	> 1.5 Kbp	Repressed	Repressed	Too Far	Shi *et al.*, 2004
M4 AChR/CHRM4	> 4 Kbp	Repressed	Repressed	Too Far	Shi *et al.*, 2004
SCN1A	> 50 Mbp	Repressed	Repressed	Too Far	Shi *et al.*, 2004
Reported LSD1 Target Sites Regulating Genes with Different Transcription Status in MCF7 Cells					
PSA	Overlapping	Activated/Repressed	Repressed	LSD1$^-$	Metzger *et al.*, 2005
c-FOS	Overlapping	Repressed	Activated	LSD1$^+$	Hakimi *et al.*, 2003

B

Relative Genomic Localization of DSL Probes and Reported LSD1 Binding Sites





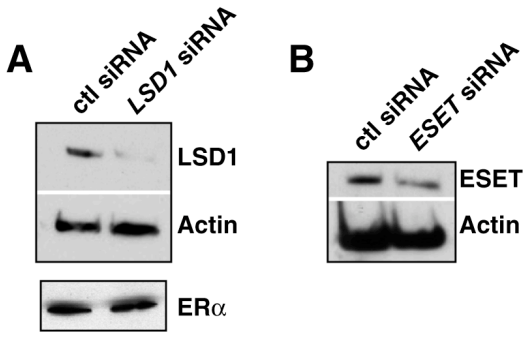
GNF Tissues (From left to right, above)

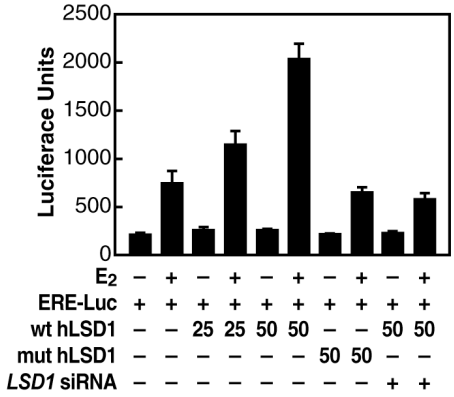
I	Whole Blood	leukemiapromyelocytic (h160)	Adipocytes
	BM-CD33+Myeloid	lymphomaburkittsDaudi	Uterus
	PB-CD14+ Monocytes	Leukemiachronicmyelogenous (k562)	Lung
	PB-BDCA4+Dentritic Cells	BM-CD71+Early erythroid	Prostate
	PB-CD4+T cells	Thymus	Thyroid
	PB-CD8+T cells	Tonsil	Fetal lung
	PB-CD19+B cells	Lymphnode	Placenta
	BM-CD105+Endothelial	Bone marrow	Fetal thyroid
	BM-CD34+	Colorectal adenocarcinoma	Pancreas
	721 B lymphoblasts	Cardiac myocytes	Pancreatic islets
	Leukemialymphoblastic (molt 4)	Smooth muscle	
	lymphomaburkittsRaji	Bronchial epithelial cells	
II	Cerebellum peduncles	Medulla oblongata	Hypothalamus
	Cerebellum	Cingulate cortex	Thalamus
	Globus pallidus	Caudate nucleus	Fetal brain
	Subthalamic nucleus	Whole brain	Spinal cord
	Temporal lobe	Amygdala	Olfactory bulb
	Pons	Prefrontal cortex	Pituitary
	Parietal lobe	Occipital lobe	
III	Testis	Adrenal gland	Ciliary ganglion
	Testis germ line	Adrenal cortex	Atrio venricular node
	Testis leydig cell	Salivary gland	DRG
	Testis intersticial	Trachea	Uterus corpus
	Testis seminiferous tubule	Ovary	Skeletal muscle
	Fetal liver	Appendix	Tongue
	Heart	Trigeminal ganglion	
	Liver	Superior cervical ganglion	
	Kidney	Skin	

A

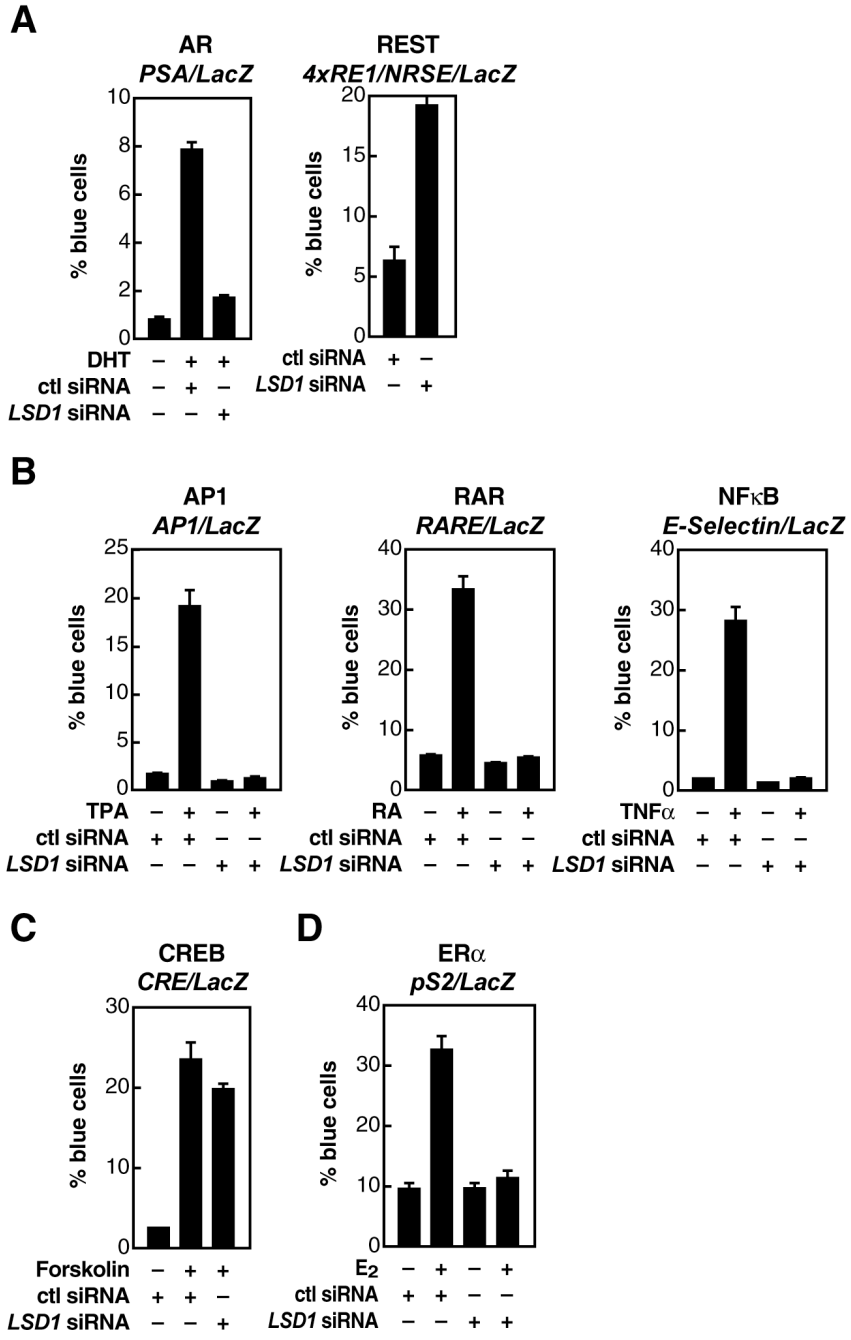
Partial list of LSD1+ that were known or prediced REST regulated genes

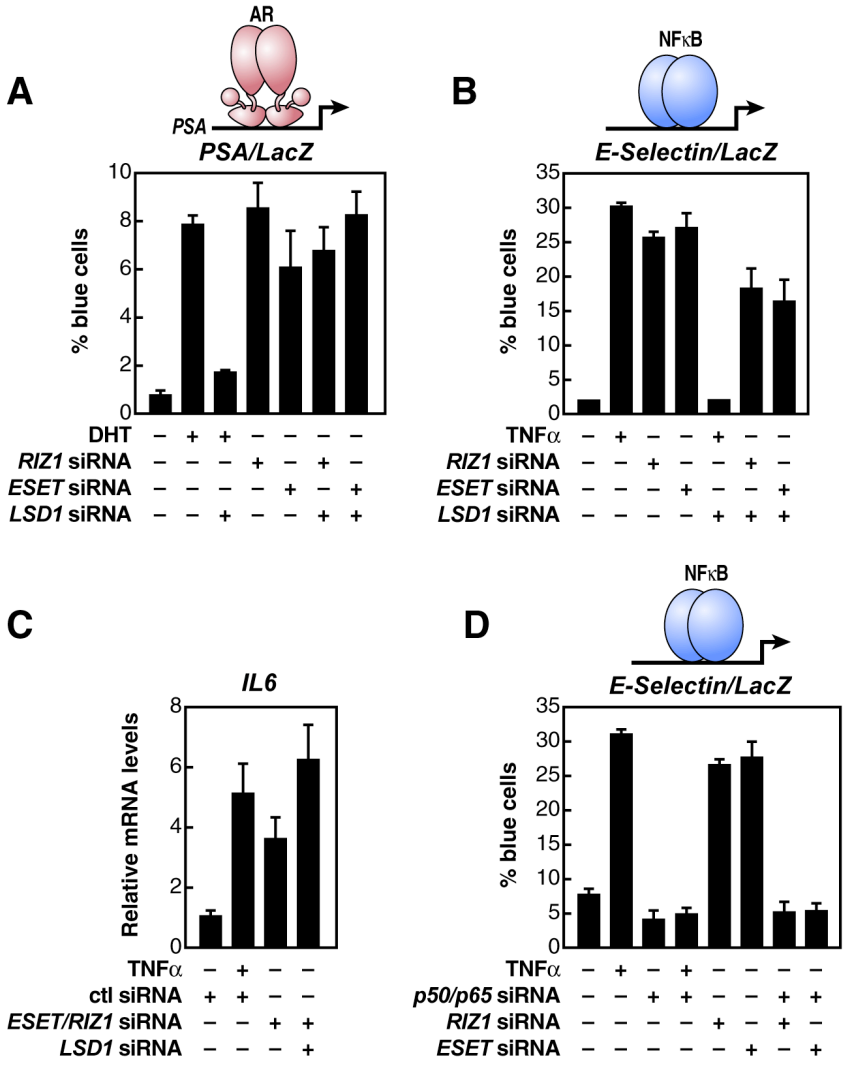
SCN3A	Brain II voltage-gated Na channel
SYN1	Synapsin I
PAX4	Paired box gene 4
CX36	Connexin 36
CRH	Corticotropin releasing hormone
DNM1	Dynamin 1
HTR6	Serotonin receptor 6
GRID1	Glutamate ionotropic δ 1 receptor
SYT2	Synaptotagmin II
KC NMA1	K-large conductance Ca-activated channel
BAI1	Brain-specific angiogenesis inhibitor 2
PLXND1	Plexin D1
NCAM2	Neural cell adhesion molecule 2
LRRN6A	Leucine rich repeat neuronal 6A
KIA1893	Inducer of neurite outgrowth 1
MLK2	Mixed lineage kinase 2
NOVA1	Neuro-oncological ventral antigen 1
NMU	Neuromedin U
SLC1A1	Neuronal high affinity glutamate transporter
	Some other REST regulated genes P<0.0009
SNAP25	Synaptosomal-associated protein 26KDa
CALB1	Calbindin 28K
NPTXR	Neuronal pentraxin receptor
SCG10	Superior cervical ganglion-10


A
ctl siRNA
LSD1 siRNA
LSD1
Actin
ERα
B
ctl siRNA
ESET siRNA
ESET
Actin



Garcia-Bassets et al., 2006 Figure S5


A
AR
PSA/LacZ
% blue cells
DHT
ctl siRNA
LSD1 siRNA
REST
4xRE1/NRSE/LacZ
ctl siRNA
LSD1 siRNA
B
AP1
AP1/LacZ
TPA
RAR
RARE/LacZ
RA
NFκB
E-Selectin/LacZ
TNFα
C
CREB
CRE/LacZ
Forskolin
D
ERα
pS2/LacZ
E2



Garcia-Bassets et al., 2006 Figure S7