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Supplemental Information

Oct4-Mediated Inhibition of Lsd1 Activity Promotes the Active and Primed State of Pluripotency Enhancers

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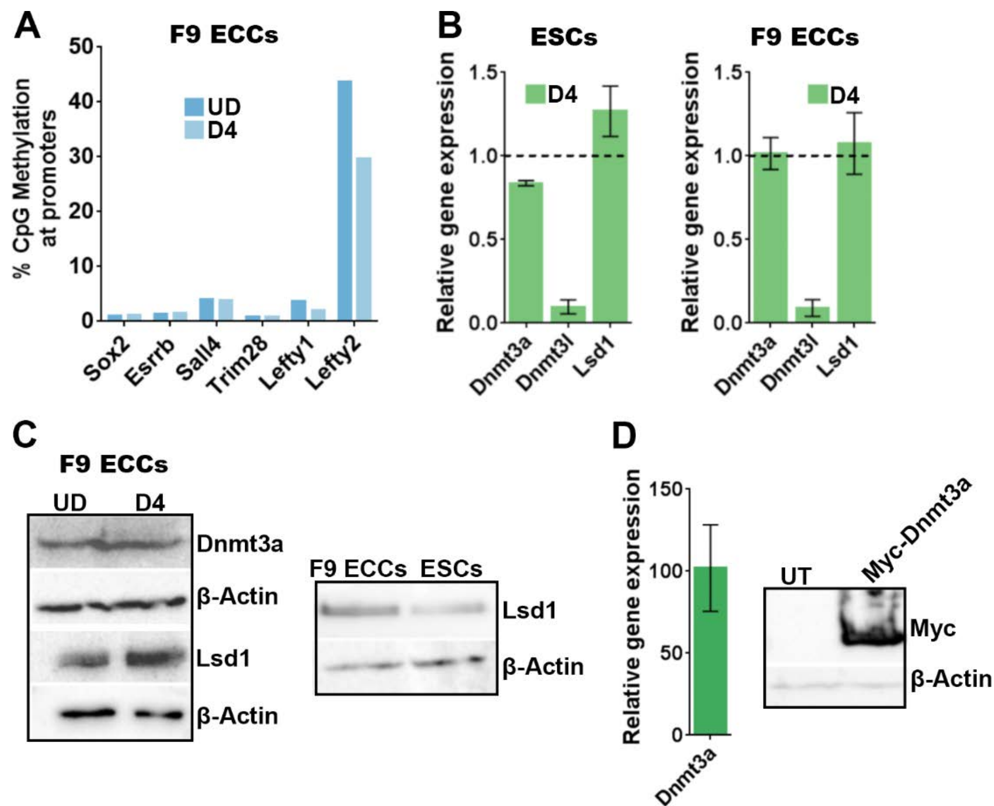


Figure S1. Expression of epigenetic effectors in embryonal carcinoma cells. (**Related to Figure 1)**

UD: undifferentiated, D4: Days post-differentiation. ESCs: embryonic stem cells and F9 ECCs: F9 embryonal carcinoma cells; PpGe: pluripotency gene enhancers.

(A) Bis-Seq analysis of DNA methylation at PpG promoters in F9 ECCs pre- and post-differentiation. DNA methylation at PpG promoters remained under 10% except *Lefty2*, which shows very high methylation in the UD state that is reduced post-differentiation.

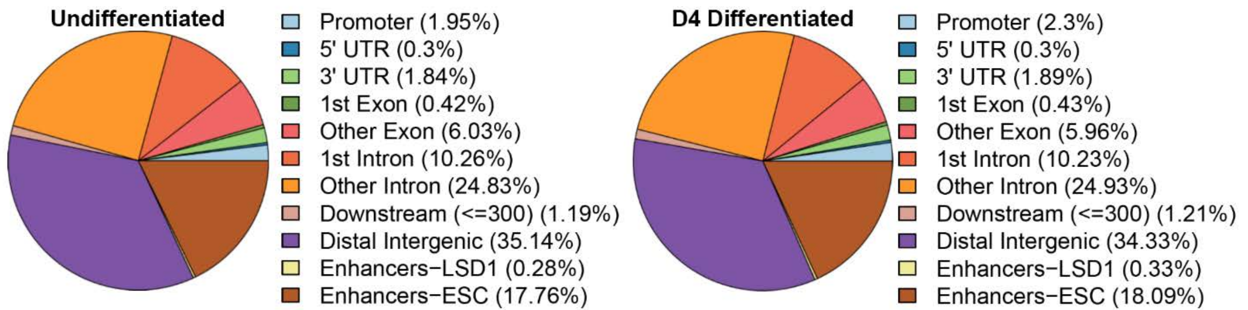
(B) RT-qPCR comparing the changes in expression of DNA methyltransferase Dnmt3a and histone demethylase Lsd1 in ESCs with that in F9 ECCs pre- and post-differentiation. The C_t values are normalized to *Gapdh* and represented relative to expression in undifferentiated cells (dotted line). In both ESCs and ECCs, Lsd1 and Dnmt3a expression is maintained post-differentiation.

(C) Western blot. A total of 50 μg of total protein from undifferentiated and differentiated cells was loaded in each well. Left panel confirms comparable expression of Dnmt3a and Lsd1 pre- and post-differentiation in F9 ECCs. Right panel compares Lsd1 expression in F9 ECCs with ESCs showing very similar levels in these cells. β-Actin is the loading control.

(D) Gene expression analysis by RT-qPCR and Western blot confirming recombinant Myc-Dnmt3a overexpression in F9 ECCs 24 hr post-differentiation (48 hr post-transfection). The data are normalized to a *Gapdh* control and shown relative to untransfected that is set to 1. β-Actin is used as loading control for Western blot.

A

Distribution of MethylRAD peaks in F9 ECCs



B

Level of DNA methylation at PpG enhancers in F9 ECCs

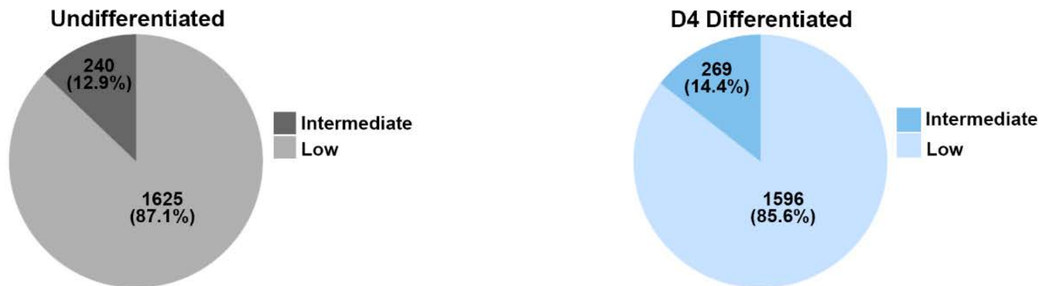


Figure S2. Distribution of MethylRAD peaks. (Related to Figure 2)

(A) Fractional distribution of MethylRAD peaks in undifferentiated and differentiated F9 ECCs across regulatory regions of the genome.

(B) Pie charts show the level of methylation at PpGe in undifferentiated (grey) and differentiated day 4 (blue) samples. Most PpGe had low levels of methylation or to a lesser extent, intermediate levels of methylation, with none having high CpG methylation.

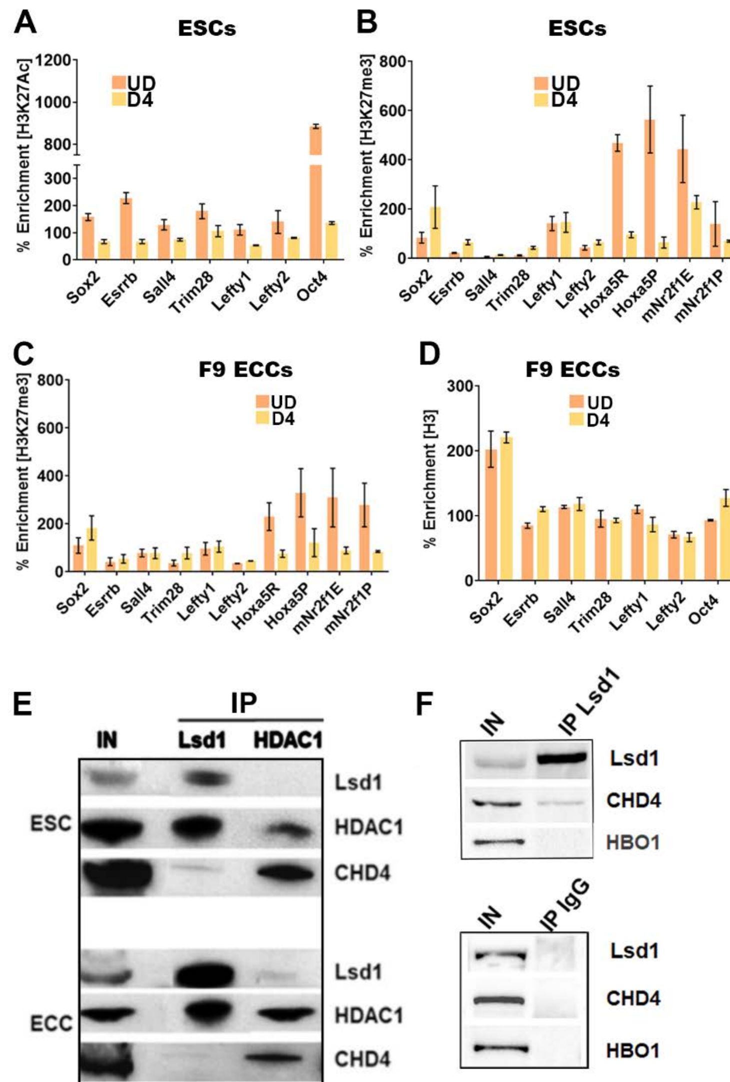


Figure S3. H3K27 modification of pluripotency gene enhancers in embryonic stem cells and embryonal carcinoma cells. (Related to Figure 3)

UD: undifferentiated; D4: Days post-induction of differentiation; IP: immunoprecipitation

(A, B, C and D) ChIP-qPCR was used to determine the enrichment of histone modifications at PpGe. (A) H3K27Ac in ESCs (B) H3K27me3 in ESCs (C) H3K27me3 and (D) H3 in F9 ECCs pre- and post-differentiation. Whereas deacetylation of PpGe is observed as a decrease in H3K27Ac signal post-differentiation in ESCs, there is no gain of H3K27me3 at these sites,

neither in ESCs nor in F9 ECCs. As previously reported, we observed a decrease in H3K27me3 at the enhancers and promoters of *Hoxa5* and *mNr2f1* genes, consistent with their transcriptional activation in response to differentiation (Laursen, Mongan et al. 2013). No change was observed in H3 occupancy between UD and D4 at PpGe. % Enrichment = Fold enrichment over input X100

(E, F) Co-IP was performed with anti-Lsd1 or anti-HDAC1 and a control IgG using whole cell extracts from undifferentiated ESCs and F9 ECCs. 20% of the input and eluate from Co-IP were probed for Lsd1-Mi2/NuRD subunits (Lsd1, HDAC1 and CHD4) on Western blot and HBO1 was used as a negative control.

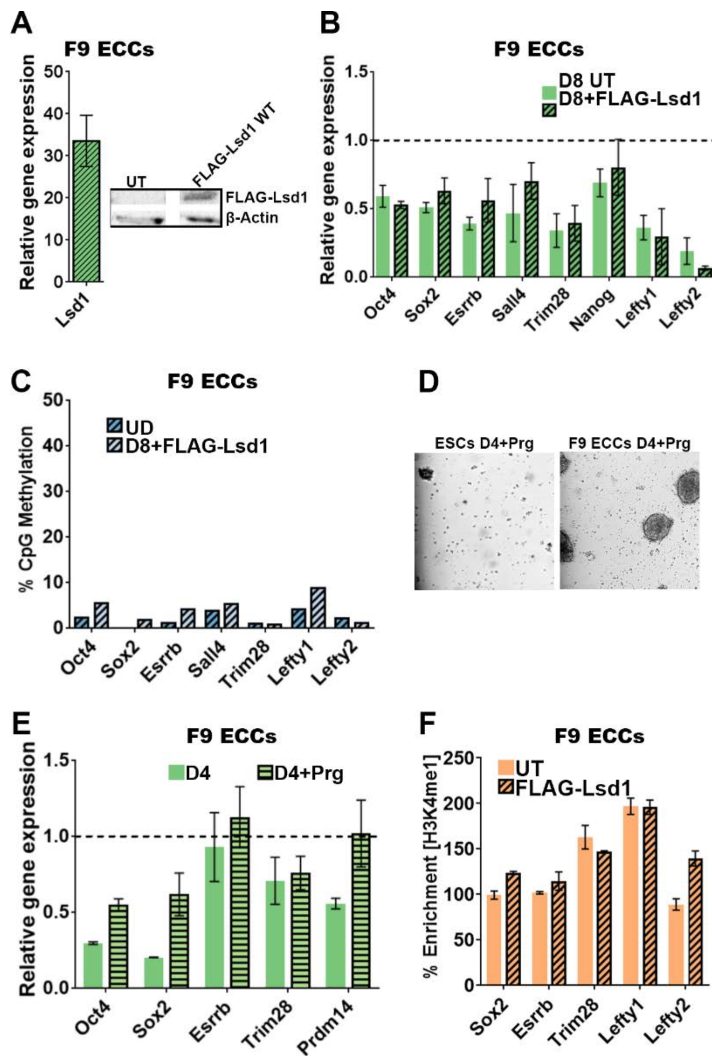


Figure S4. Effect on Lsd1 on differentiation and pluripotency gene enhancer silencing in embryonal carcinoma cells. (Related to Figure 3)

UD: undifferentiated; D8: Days post-induction of differentiation; Prg: pargyline.

(A) Gene expression analysis by RT-qPCR and Western blot examining the expression of recombinant FLAG-Lsd1 in F9 ECCs 24 hr post-differentiation (48 hr post-transfection). The C_t values are normalized to *Gapdh* and represented relative to expression in untransfected cells (set to 1). β-Actin is used as loading control for Western blot.

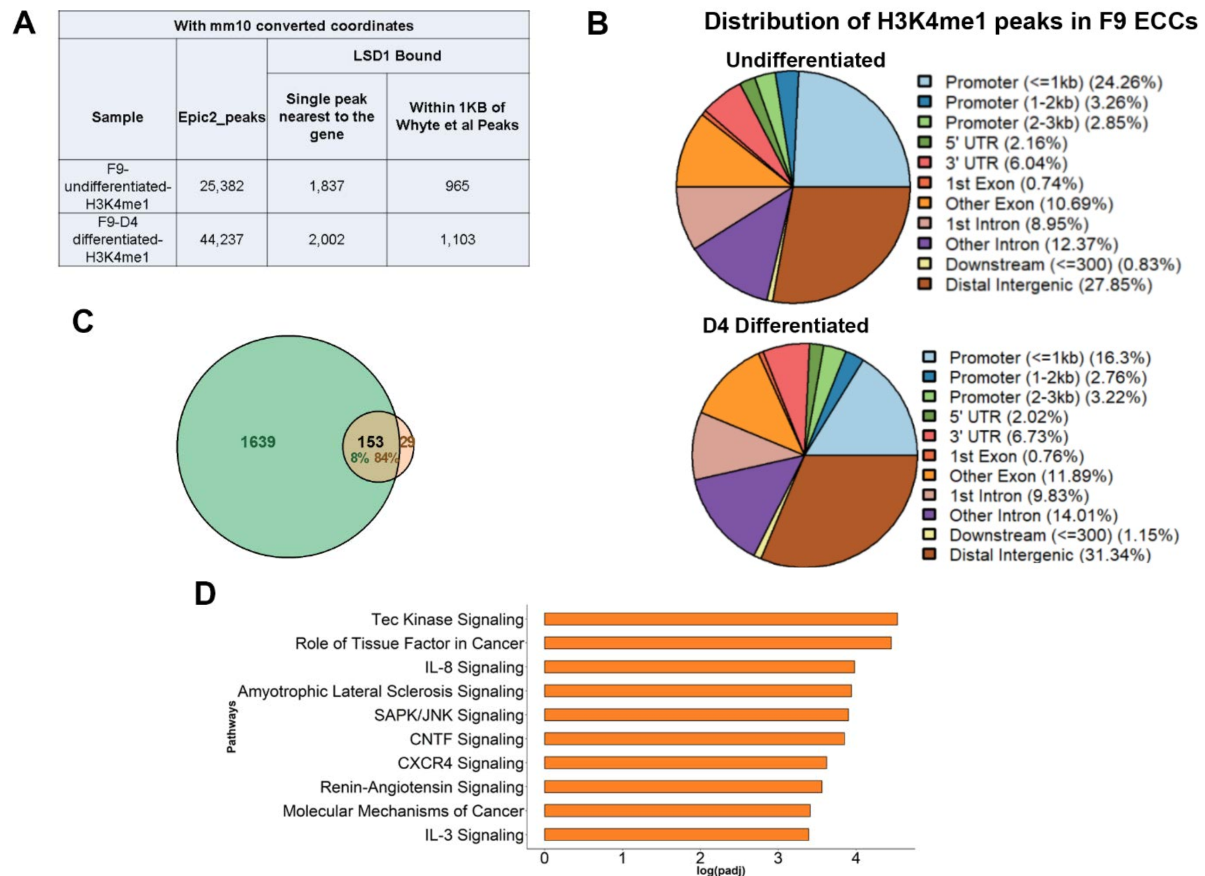
(B) Gene expression analysis by RT-qPCR of PpGs in F9 ECCs expressing FLAG-Lsd1. Similar to our observation in untransfected WT F9 ECCs, PpGs are partially repressed in these cells, showing no effect of recombinant Lsd1 on PpG repression (p-value >0.1).

(C) Bis-Seq analysis of DNA methylation at PpGe in F9 ECCs expressing recombinant FLAG-Lsd1 showed no gain, corresponding to no loss of H3K4me1 at these sites. Data are an average and SEM of two biological replicates.

(D) Bright field microscopy of ESCs and ECCs differentiated for 4 days in the presence of the Lsd1 inhibitors Prg. 80-90% cell death is observed in ESCs. Lsd1 inhibitor has no effect on F9 ECC differentiation as shown by a normal morphology of embryoid bodies. Scale bar is 100 µm.

(E) Gene expression analysis of PpGs by RT-qPCR in differentiating F9 ECCs untreated or treated with Prg. A slight derepression of some PpGs was observed in inhibitor treated cells post-differentiation. Gene expression was normalized to *Gapdh* and represented as relative change to gene expression in undifferentiated (dotted line).

(F) Undifferentiated F9 ECCs were transfected with FLAG-Lsd1 and cultured for 72 hr. H3K4me1 enrichment was determined using ChIP-qPCR. No change in H3K4me1 levels was observed compared to untransfected (UT) cells. % Enrichment = Fold enrichment over input X100



With mm10 converted coordinates			
		LSD1 Bound	
Sample	Epic2_peaks	Single peak nearest to the gene	Within 1KB of Whyte et al Peaks
F9-undifferentiated-H3K4me1	25,382	1,837	965
F9-D4 differentiated-H3K4me1	44,237	2,002	1,103

Figure S5. Distribution of H3K4me1 ChIP-Seq peaks. (Related to Figure 4)

(A) Summary of the overlapping LSD1 bound sites between H3K4me1 peaks in F9 ECCs and ESCs. To determine correct overlap, peak coordinates from Whyte.et al were converted from mm9 to mm10 using CrossMap tool.

(B) Fractional distribution of H3K4me1 peaks in undifferentiated and differentiated F9 ECCs throughout the genome.

(C) Overlap of the 182 sites that show decrease in H3K4me1 post-differentiation in F9 ECCs with those in ESCs (See Supplementary methods).

(D) Top ten most statistically significant enriched canonical pathways amongst the genes associated with decrease in F9 ECCs.

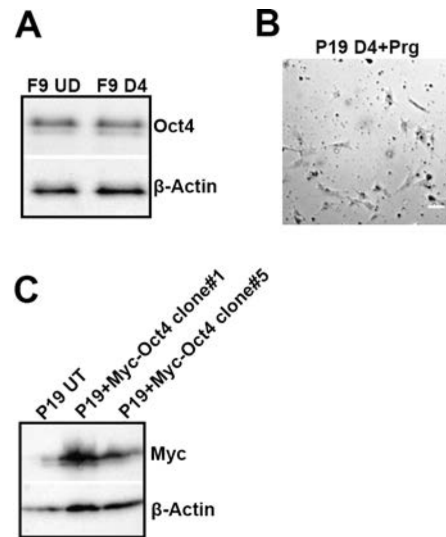


Figure S6. P19 embryonal carcinoma cells are sensitive to Lsd1 inhibition. Related to Figure 5 and Figure 6

D4: Days post-induction of differentiation; Prg: pargyline; UT: untransfected control.

(A) Western blot showing protein levels of Oct4 in undifferentiated and D4 differentiated F9 ECCs. A continued expression of Oct4 is seen in differentiated cells although it is lower than that observed in the undifferentiated cells. β-Actin was used as a loading control.

(B) Bright field microscopy of P19 ECCs differentiated for 4 days in presence of Lsd1 inhibitor, Prg. 80-90% cell death is observed in P19 ECCs indicating that Lsd1 activity at PpGe is required for differentiation. Scale bar is 100 μm.

(C) Expression analysis by Western blot confirming recombinant Myc-Oct4 overexpression in two independent clones of P19 ECCs. β-Actin was used as a loading control for Western blot.

Supplementary Tables

Genomic element		No. of CpGs
Enhancer	Lefty1	4
	Lefty2	6
	Sall4	4
	Sox2	10
	Trim28	6
	Esrrb	6
	Oct4	4
Promoter	Lefty1	6
	Lefty2	9
	Sall4	10
	Sox2	18
	Trim28	19
	Esrrb	29
Imprinted region	H19	11

Table S1. Number of CpGs at each site used for Bis-Seq DNA methylation analysis. Related to DNA methylation analysis in Method Details

The number of CpG sites used to compute percent methylation within the H19 imprinted region, PpG enhancers, and promoters.

Sample	*Lefty1*	*Lefty2*	*Sall4*	*Sox2*	*Trim28*	*Esrrb*	*Oct4*	***Total***
ESCs UD	28465	21981	2861	5826	17973	6034	1902	**85042**
ESCs D4	401	275	216	881	269	5381	6322	**13745**
F9 ECCs UD	57536	49939	27259	40357	14261	22395	6322	**232670**
F9 ECCs D4	62876	38534	29204	32570	13304	21569	8865	**214866**
F9 ECCs D6	62185	38590	30187	28239	10163	13059	6980	**182422**
F9 ECCs+Myc-Dnmt3a UD	3733	2692	1308	2612	1249	24671	311	**38969**
F9 ECCs+Myc-Dnmt3a D8	2734	1637	1135	2665	1097	4316	350	**24705**
F9 ECCs+FLAG-Lsd1 UD	2513	2267	816	4204	820	24412	1791	**43102**
F9 ECCs+FLAG-Lsd1 D8	2819	1950	1946	4530	1169	21576	1601	**42022**
P19 ECCs UD	26651	18427	10541	31377	9437	46154	7044	**175098**
P19 ECCs D4	40376	30470	18198	27196	11267	56736	14506	**241010**
P19 ECCs D8	4713	8696	5880	28944	6475	24128	4498	**96640**
P19 ECCs+Myc-Oct4 UD	23029	11988	11538	9324	7735	28746	7510	**130029**
P19 ECCs+Myc-Oct4 D4	35014	20519	14300	18022	13227	43559	14989	**210333**
Total	**353045**	**247965**	**156838**	**236746**	**108446**	**342735**	**76669**	**1522445**

Table S2. Number of reads for each enhancer used for Bis-Seq analysis. Related to DNA methylation analysis in Method Details

The total number of reads from Wide-Seq runs that were used for data presented in this study. The number of reads were calculated for each sample and enhancer, along with the overall total number of reads.

Sample	*Lefty1*	*Lefty2*	*Sall4*	*Sox2*	*Trim28*	*Esrrb*	*H19*	***Total***
F9 ECCs UD	51	726	486	9556	7575	4191	11062	**33647**
F9 ECCs D4	99	1590	1400	17431	16307	13865	9376	**60068**
Total	**150**	**2316**	**1886**	**26987**	**23882**	**18056**	**20438**	**93715**

Table S3. Number of reads for promoters and imprinted regions used for Bis-Seq analysis. Related to DNA methylation analysis in Method Details

The total number of reads from Wide-Seq runs that were used in this study. The number of reads were calculated for each sample, promoter site, and imprinted locus, as well as the overall total number of reads.