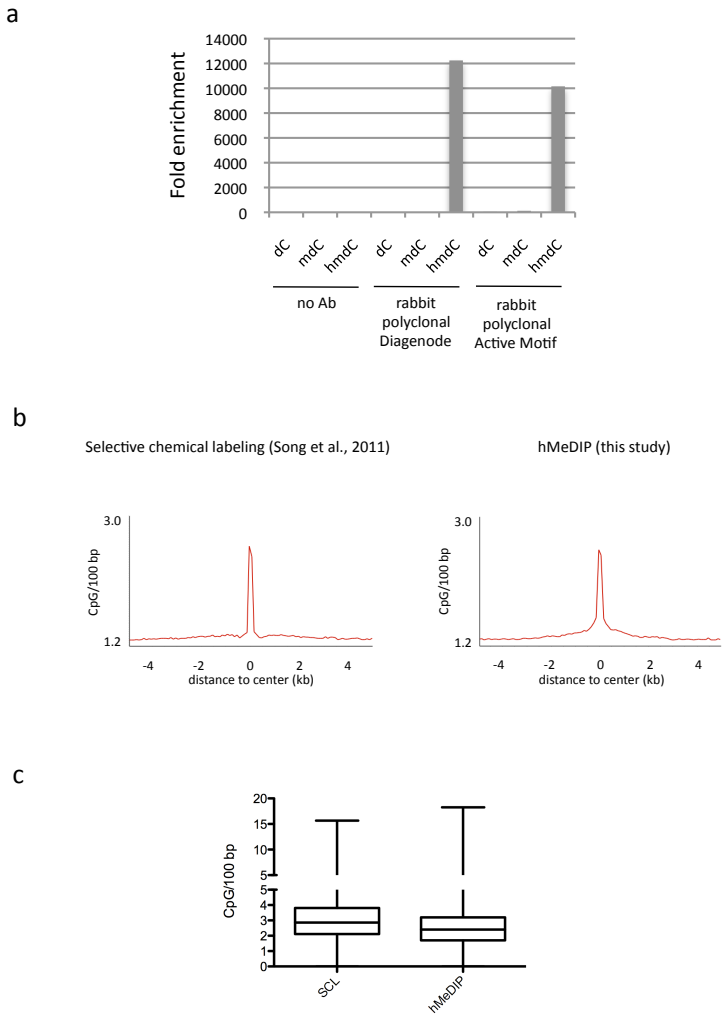

Figure S1. Specificity of the hMeDIP procedure. **(a)** One ng of C-, 5mC- or 5hmC-containing DNA amplicons were mixed to 4 μg of purified genomic DNA and submitted to the hMeDIP procedure using rabbit polyclonal antibodies either from Diagenode or from Active Motif. Recovered DNA was analyzed by qPCR. Data are plotted as fold enrichment relative to « no antibody » conditions. Amplicons were generated by PCR from human genomic DNA with dC, 5mdC or 5hmdC and covered 541 bp from the *ps2* promoter containing 146 cytosines on one strand and 127 cytosines on the other strand. **(b)** Analysis of CpG density in 5hmC called peaks (38,584 regions) from RA-treated P19 cells compared with 5hmC called peaks (32,646 regions) from adult male mouse cerebellum DNA recovered by selective chemical labeling (24, GSM23645). Both average profiles **(b)** and box plots **(c)** are shown. Average profiles were obtained by counting the number of CpGs every 100 bp of 10 kb of sequence flanking the center of peaks. Box plots show the distribution of the number of CpGs/100 bp in called peaks and indicate minimum and maximum values.

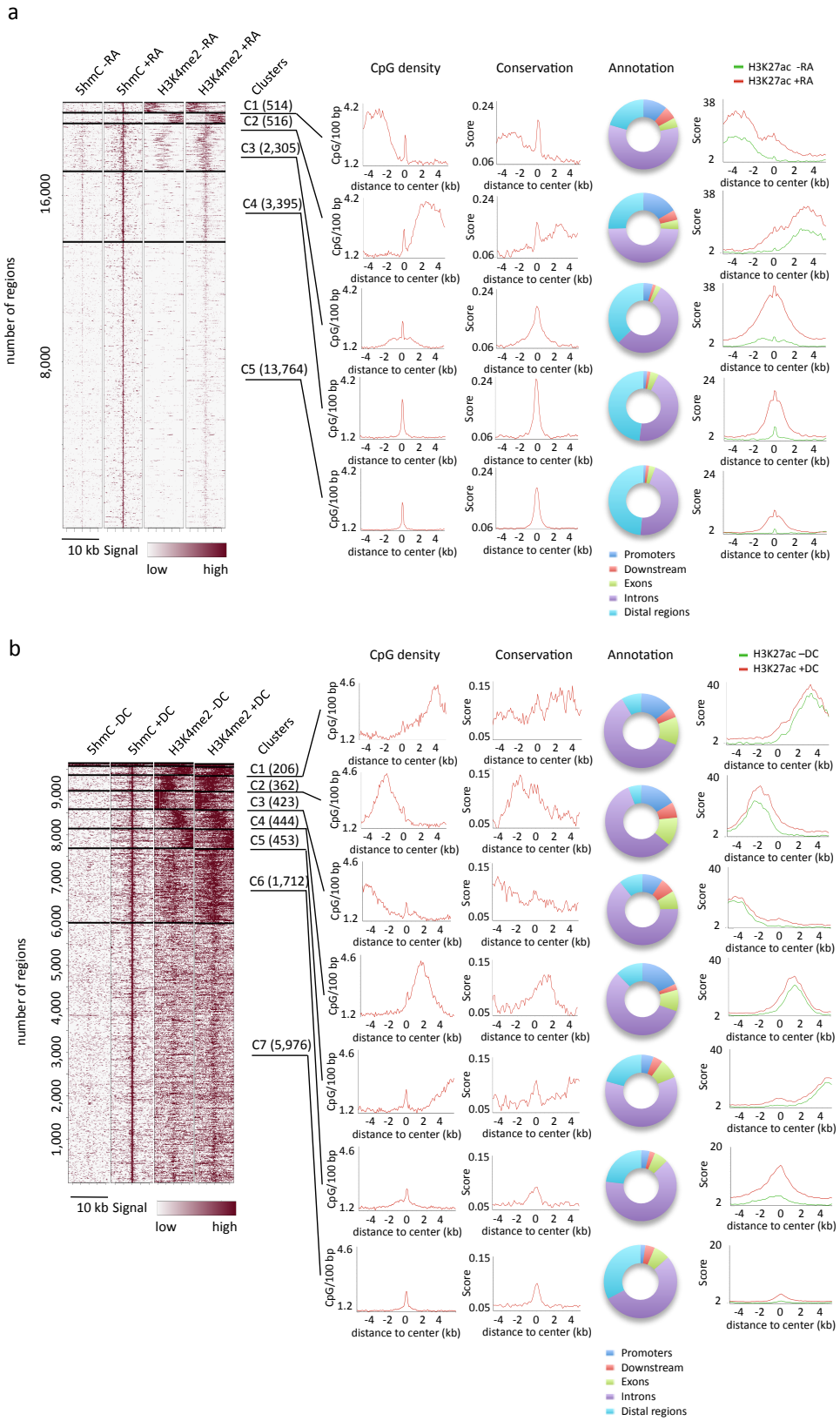

Figure S2. Characterization of differentiation-dependent hydroxymethylated genomic regions in P19 **(a)** and 3T3-L1 **(b)** cells. Heatmaps represent 5hmC and H3K4me2 profiles in clusterized 5hmC-up regions. Clustering was run taking into account all signals. Horizontal lines demarcate the different clusters and the number of regions lying within each cluster is given in brackets. Average profiles of CpG density, conservation among vertebrates and H3K27ac were generated for each cluster of 5hmC-up regions. Gene-centered annotation of clusters is depicted as rings.

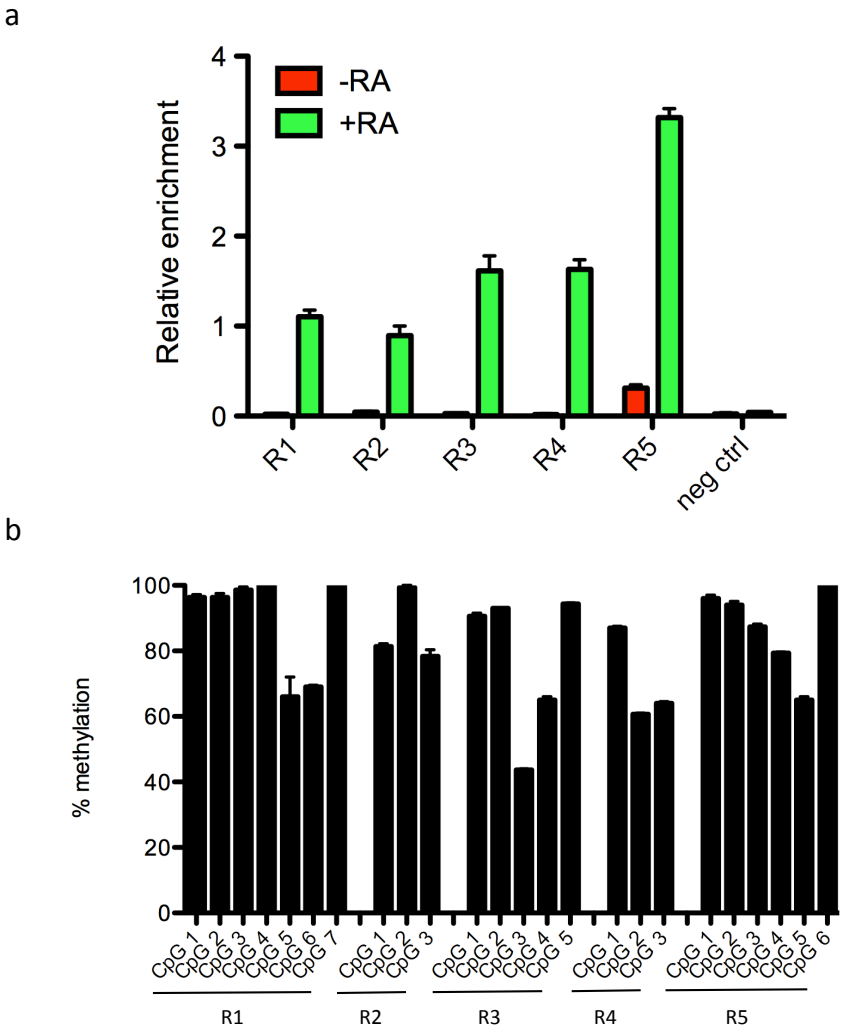

Figure S3. (a) Measurement of 5hmC levels in enhancers R1, R2, R3, R4 and R5 by selective chemical labeling. Genomic DNA (500 ng) from P19 cells was glucosylated *in vitro*, coupled to biotin and purified with streptavidin-coated magnetic beads according to the « Hydroxymethyl Collector » kit procedure (Active Motif). Purified DNA was analyzed by qPCR. Data are shown as mean +/- SEM (n=3) showing enrichment relative to input DNA. **(b)** Quantification of the methylation status of R1 to R5 enhancers in undifferentiated P19 cells. Pyrosequencing of bisulfite-modified genomic DNA allowed to quantify the methylation level of CpGs located in the vicinity of the center of hmCup regions.

a

	All hmCup sites		Intergenic hmCup sites		Intronic hmCup sites		Random
	P19	3T3	P19	3T3	P19	3T3	
RANK	Name/Family	Name/Family	Name/Family	Name/Family	Name/Family	Name/Family	
1	PBX	NF1	PBX	NF1	PBX	NF1	HMGIY
2	*SP1*	PPARg	*SP1*	CEBP	*SP1*	PPARg	*MINI*
3	EGR	CEBP	*MINI*	HEN	EGR	CEBP	PIT1
4	*MINI*	AP4	EGR	PPARg	CACCC	HEN	GATA
5	CACCC	HEN	WT1	*SP1*	*MINI*	CREB	*ZNF219*
6	*ZNF219*	*EBOX*	AP4	AP4	*ZNF219*	AP4	*PAX*
7	GGG	CREB	CACCC	*MINI*	GGG	MYOGNF1	TGTGGT
8	WT1	MYOGNF1	*ZNF219*	CREB	WT1	*SP1*	*OLF1*
9	RREB	*SP1*	GGG	*EBOX*	TEF	*EBOX*	FOX
10	TEF	*MINI*	RREB	RREB	AP2	*MINI*	*LMAF*
11	AP2	EBF	*LMAF*	MYOGNF1	RREB	EBF	*AR*
12	HEN	AP2	HNF4	ETS	AP4	CACCC	*EBOX*
13	AP4	*AR*	AP2	MIF1	HEN	MTF1	*ATCGAT*
14	COUP-TF	EGR	TEF	WT1	*LMAF*	*AR*	MEF2
15	*EBOX*	RREB	MTF1	SPZ	TFIII	*PAX*	SOX
16	*LMAF*	CACCC	MEIS1	TEF	MOVO	AP2	NFKB
17	TFIII	MIF1	*EBOX*	CACCC	VDR	*OLF1*	OCT
18	CREB	ETS	TFIII	NRF	*EBOX*	TFIII	ZBRK1
19	MOVO	*PAX*	AP1	*AR*	CREB	ETS	*SP1*
20	MEIS1	SMAD	MOVO	SREB	*ATCGAT*	HIC1	CMAF

P19 specific
3T3 specific
common

b

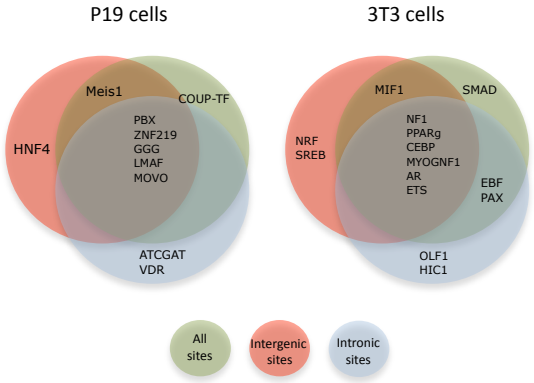

Figure S4. Transcription factor binding motifs enriched in 5hmC-up regions from P19 and 3T3-L1 cells. Motif search was run with CENTDIST either on all 5hmC-up regions or on regions classified as intergenic or intronic in both cell lines. 20,494 random regions were also analyzed for the presence of motifs. **(a)** Table indicating enriched motifs ranked according to increasing *P* value. Motifs specific for P19 cells have been highlighted in red and motifs specific for 3T3-L1 cells in green. Motifs also found in random regions are in italics. **(b)** Venn diagram showing the overlap between motifs enriched in intergenic, intronic and all regions.

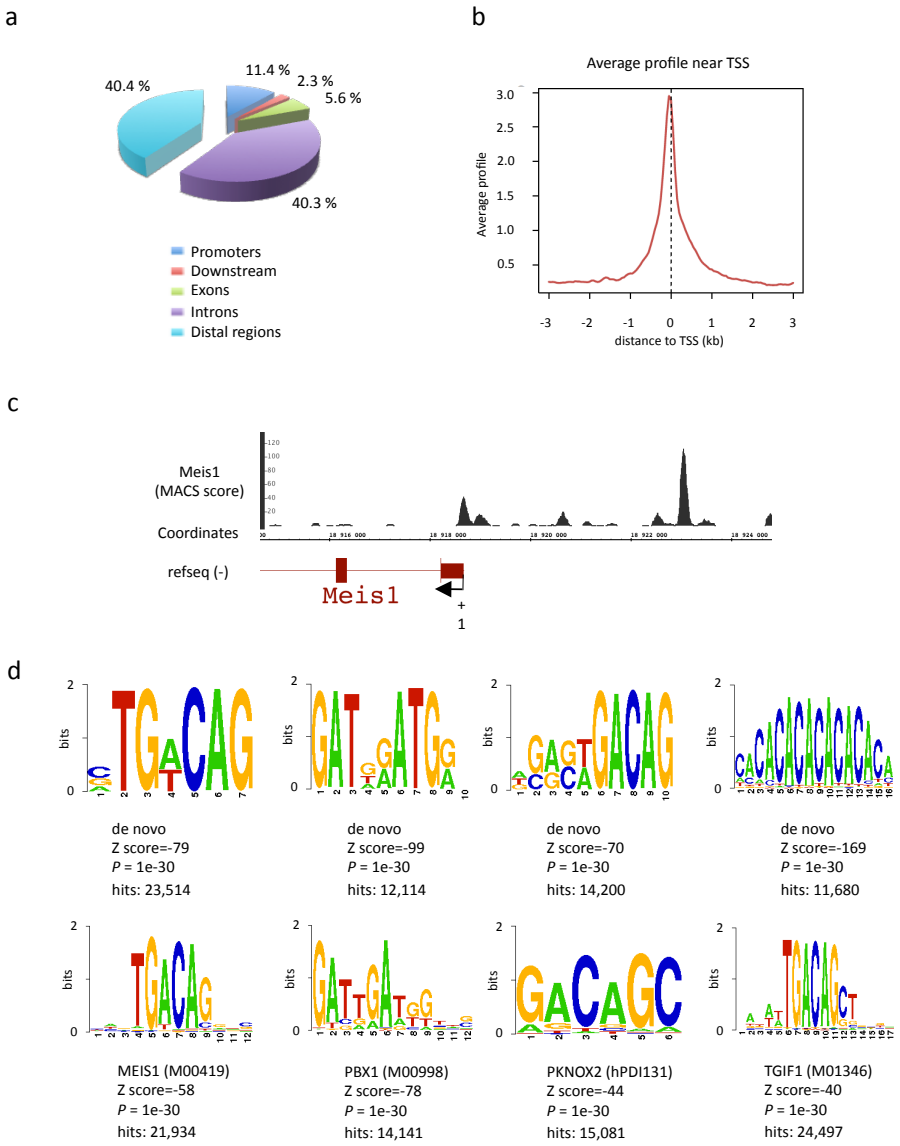

Figure S5. ChIP-seq analysis of Meis1 binding events in RA-treated P19 cells. (**a**) Pie chart indicating the percentage of distribution of Meis1 peaks relative to specific gene features (promoters: -3 kb to TSS, downstream: TTS to +3 Kb). Meis1 ChIP-seq reads were enriched in promoters (P=9.3e^{-62}) and 5'UTR (P=3.4e^{-42}) as compared to an expected random distribution. **(b)** Average profile of Meis1 binding near transcription start sites (TSS). (**c**) Meis1 binds to its own gene promoter. Integrated genome browser representation of Meis1 ChIP-seq signal at the promoter and 5' region of *meis1*. (**d**) Transcription factor motifs enriched in Meis1 ChIP-seq reads. Regions binding Meis1 were scanned for transcription factor recognition motifs with the SeqPos motif tool from Cistrome (http://cistrome.dfci.harvard.edu), using *de novo* motifs search as well as Transfac and PDI matrices. SeqPos retrieved binding motifs for the three related TALE-homeodomain (HD) proteins Meis, TGIF and PKNOX, as well as for PBX (a known partner of TALE-HD proteins) with high confidence, validating our ChIP-seq data.

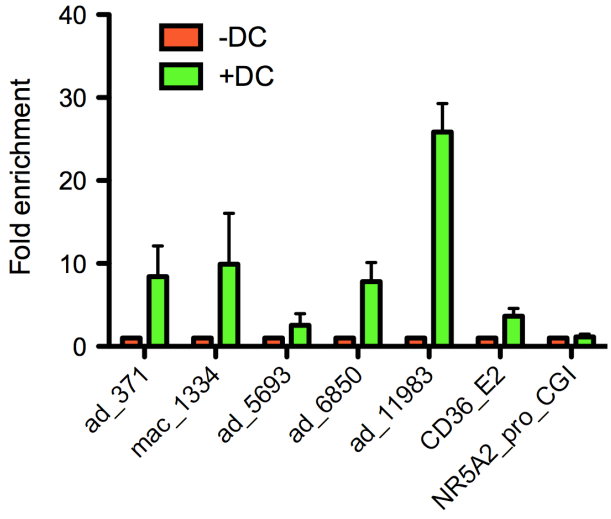

Figure S6. hMeDIP-qPCR analysis of 5hmC enrichment of randomly selected PPARγ-bound enhancers during adipocyte differentiation of 3T3-L1 cells. Enhancers were selected from Lefterova et al. (68) and from Mikkelsen *et al.* (6). The *Nr5a2* promoter served as a negative control.

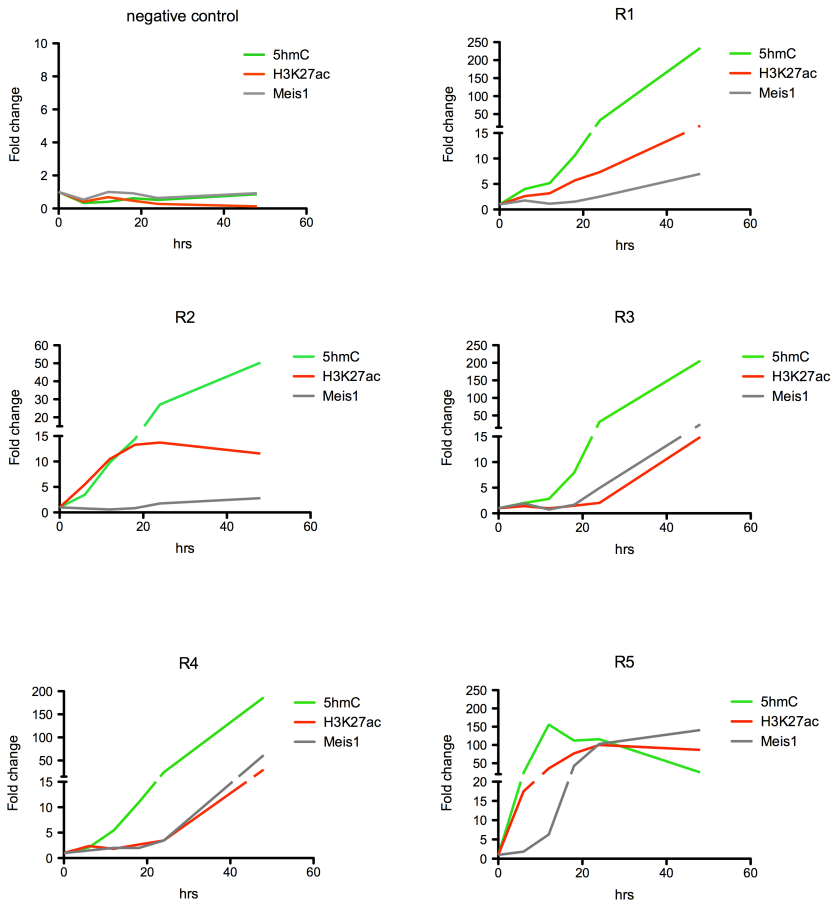

Figure S7. Kinetic analysis of 5hmC levels, H3K27 acetylation and Meis1 binding at R1 to R5 enhancers during differentiation of P19 cells. Samples were taken at 0, 6, 12, 18, 24 and 48 hrs after RA addition to the culture medium and processed for hMeDIP- or ChIP-qPCR assays. Data are shown as fold change relative to the negative control (mean values of triplicates, error bars were omitted for the sake of clarity).

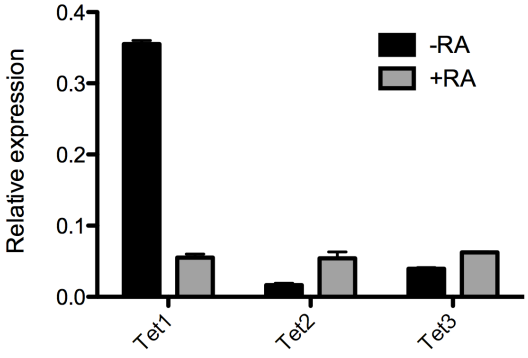

Figure S8. RT-qPCR analysis of Tet1,2 and 3 expression in P19 cells. Data are shown as mean +/- SEM (n=3) Tet expression relative to the expression of the control gene *36B4*.

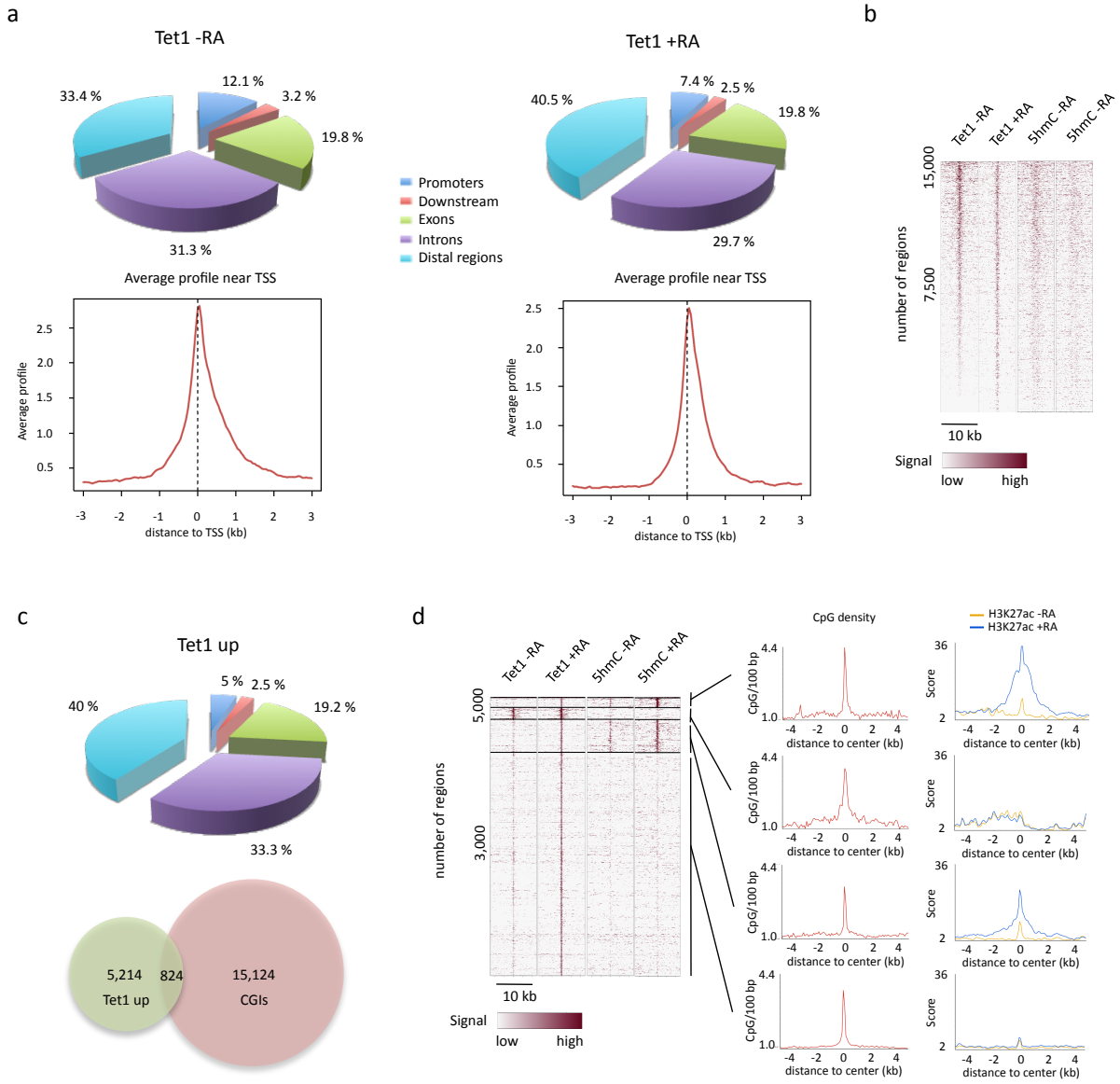

Figure S9. ChIP-seq analysis of Tet1 binding events in P19 cells (+/- RA). **(a)** Gene-centered annotation of Tet1 peaks and average profile of Tet1 binding near TSS from undifferentiated (left) and RA-treated (right) P19 cells. **(b)** Heatmap of Tet1 and 5hmC levels in 15,948 CGIs. **(c)** Gene-centered annotation of Tet1-up regions (top) and Venn diagram showing the ovelap between Tet1-up regions and CGIs. (d) Heatmap representation of Tet1 and 5hmC signals in clusters of non-CGI Tet1-up regions. Clustering was applied according to all signals. Average profiles of CpG density and H3K27ac are shown for each cluster.

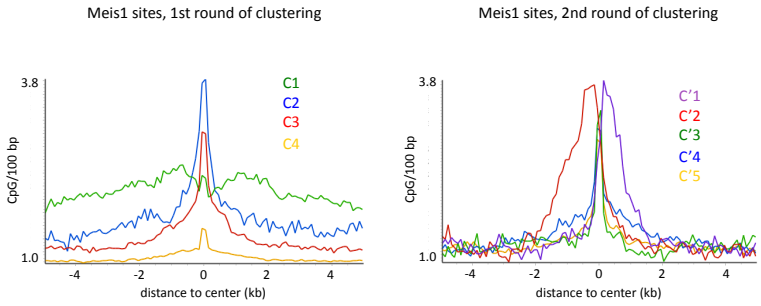

Figure S10. Average profiles of CpG density at clusters of Meis1 binding sites. CpGs were counted in 100 bp windows for 10 kb of sequence flanking the center of Meis1 peaks. Clusters are from Fig. 6.

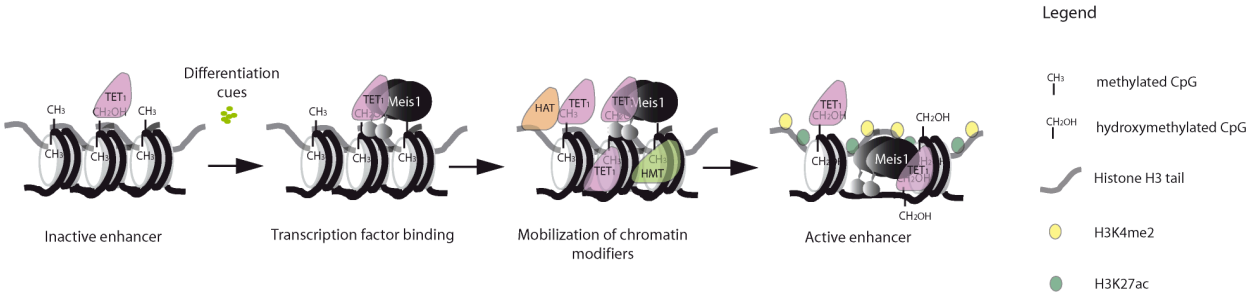

Figure S11. Model of enhancer activation through Meis1 binding to regions of low intermediate CpG density. At intermediate CpG density enhancers, Meis1 binds to Tet1-occupied regions and stimulates Tet1 recruitment and/or activity. Enhancer activity correlates with increased levels of 5hmC, H3K4me2 and H3K27ac.

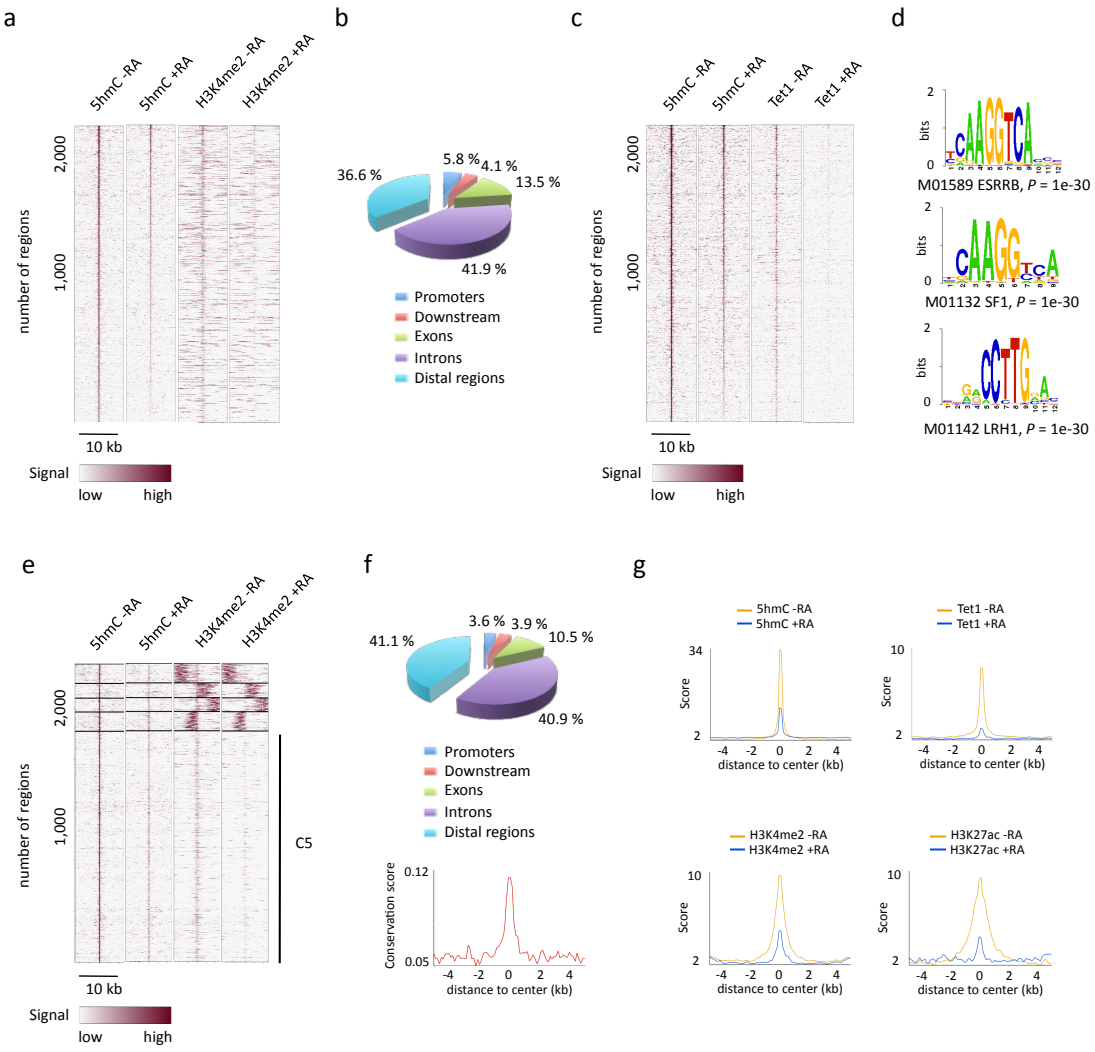

Figure S12. Loss of 5hmC correlates with chromatin de-activation. **(a)** Heatmaps of 5hmC and H3K4me2 signals in 2,350 identified 5hmC-down regions rank-ordered according to 5hmC signal from undifferentiated cells. **(b)** Gene-centered annotation of 5hmC-down regions depicted as a ring. **(c)** Heatmaps of 5hmC and Tet1 signals in 5hmC-down regions rank-ordered as in (a). **(d)** Transcription factor binding motifs identified by Seq-Pos motif tool as enriched in 500 bp around the peak center of 5hmC-down regions. **(e)** Heatmaps of 5hmC and H3K4me2 signals in 5hmC-down regions clustered according to both signals. **(f)** Gene-centered annotation depicted as a ring (top) and average profile of conservation (bottom) of 5hmC-down regions from cluster C5. **(g)** Average profiles of 5hmC, Tet1, H3K4me2 and H3K27ac signals for 5hmC-down regions from cluster C5.