Supplementary Figure S1

A

Setdb1 conditional knockout iMEF (*Matsui et al., Nature, 2010*)

↓ 4-OHT

Setdb1 knockout iMEF #3-12 (*Kato et al., Nat. Com., 2018*)

↓ *Suv39h1/h2 knockout (CRISPR-Cas9)*

Setdb1/Suv39h1/Suv39h2 triple knockout iMEF #27-34 (*Fukuda et al., Com. Biol., 2021*)

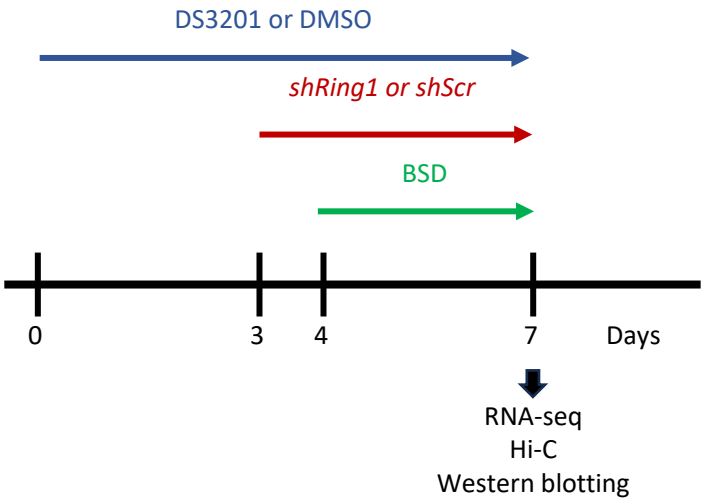
↓ *Ehmt1/2 knockout (CRISPR-Cas9)*

Setdb1/Suv39h1/Suv39h2/Ehmt1/Ehmt2 five knockout iMEF #14, #55 (*Fukuda et al., NAR, 2023*)

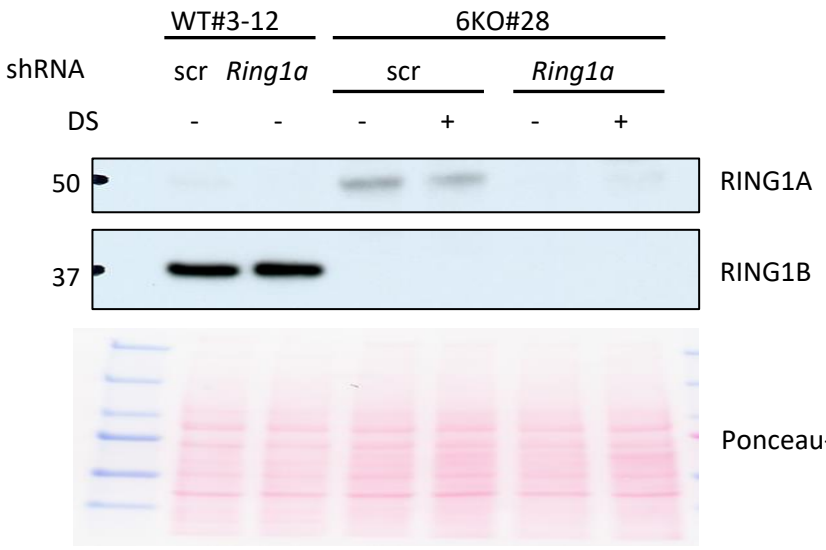
↓ *RIng1b knockout in #55 (CRISPR-Cas9)*

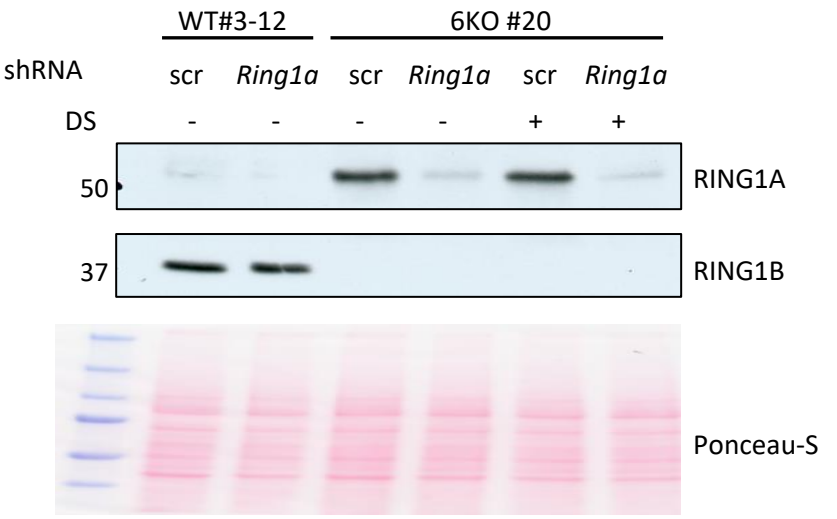
Setdb1/Suv39h1/Suv39h2/Ehmt1/Ehmt2/Ring1b six knockout iMEF #20, #28 (*Fukuda et al., NAR, 2023*)

B

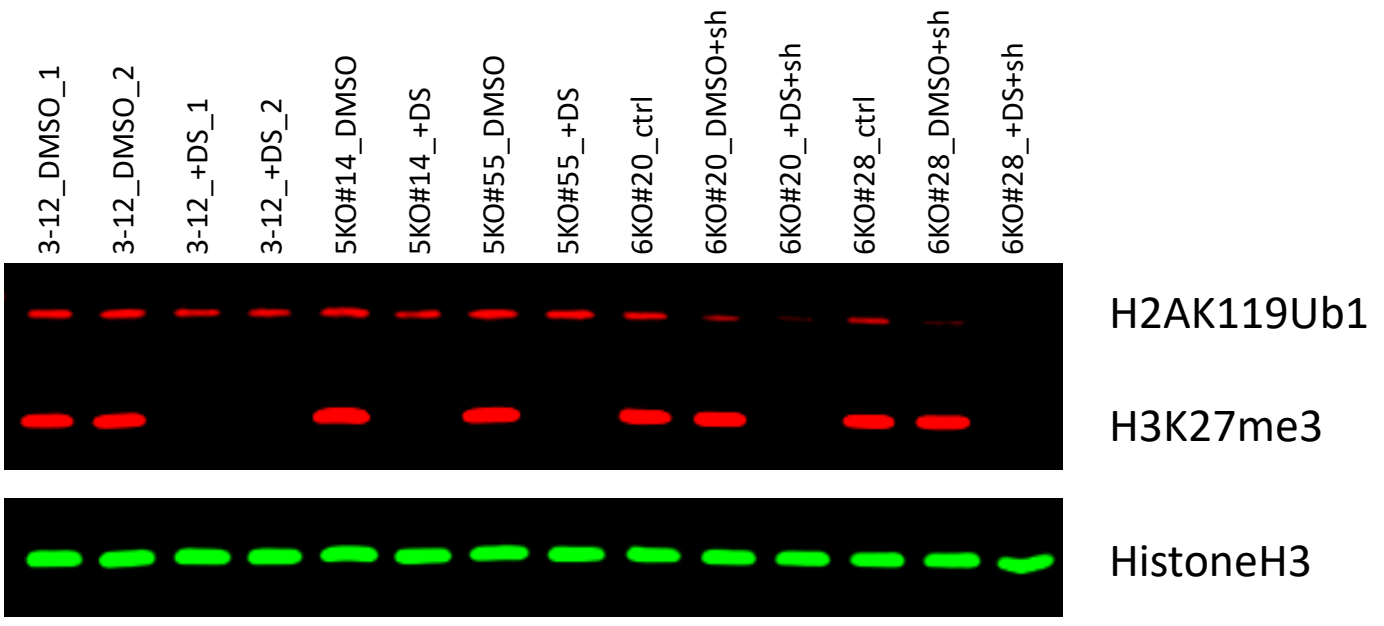


C

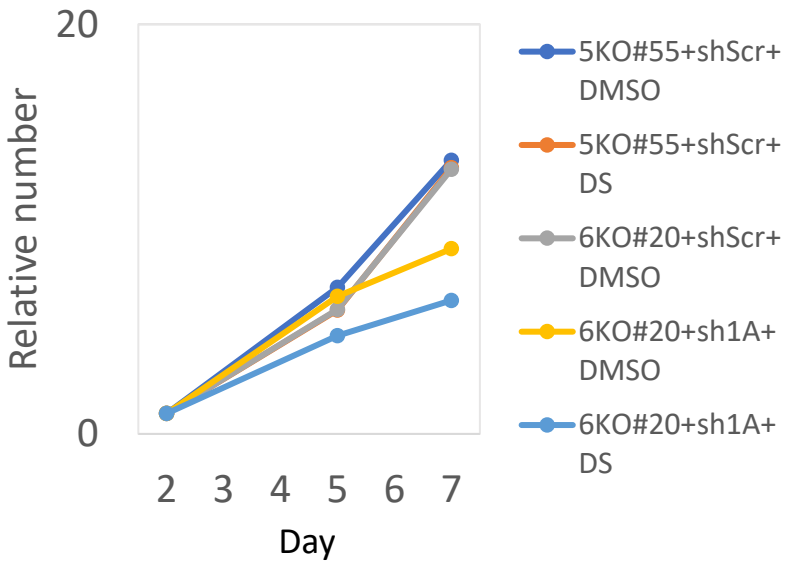


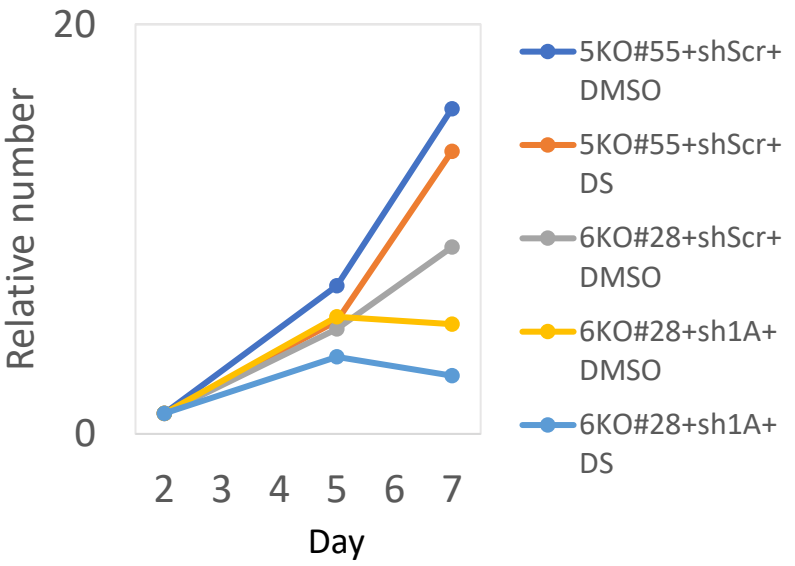


D



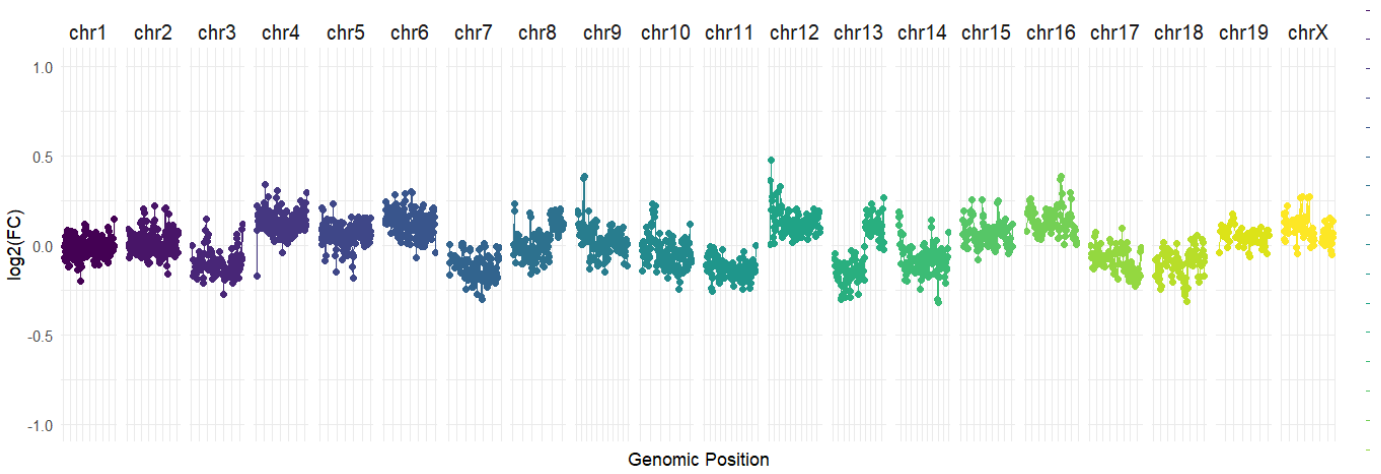
E



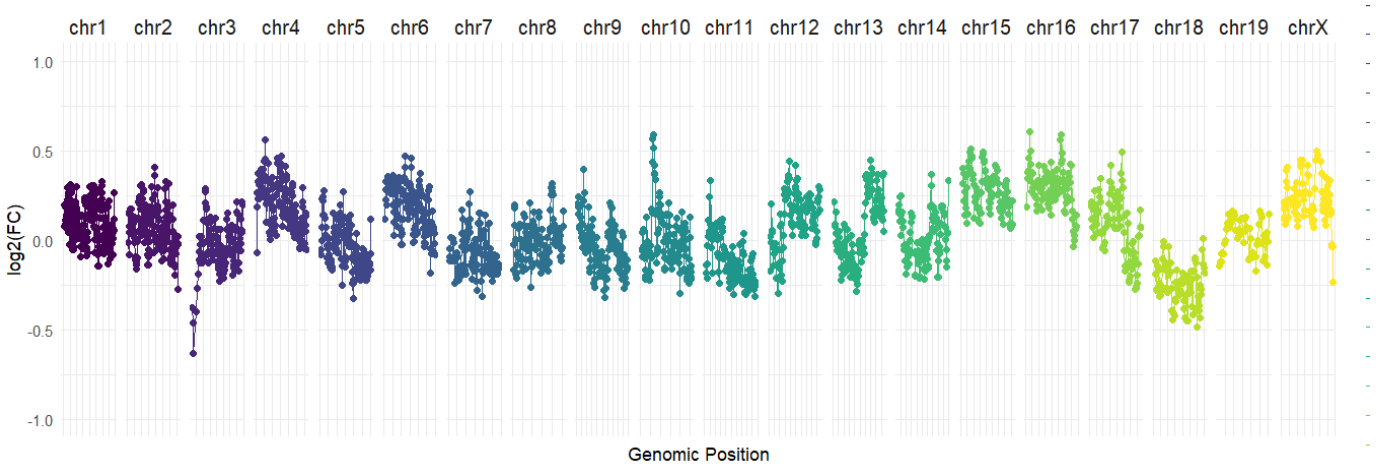


F

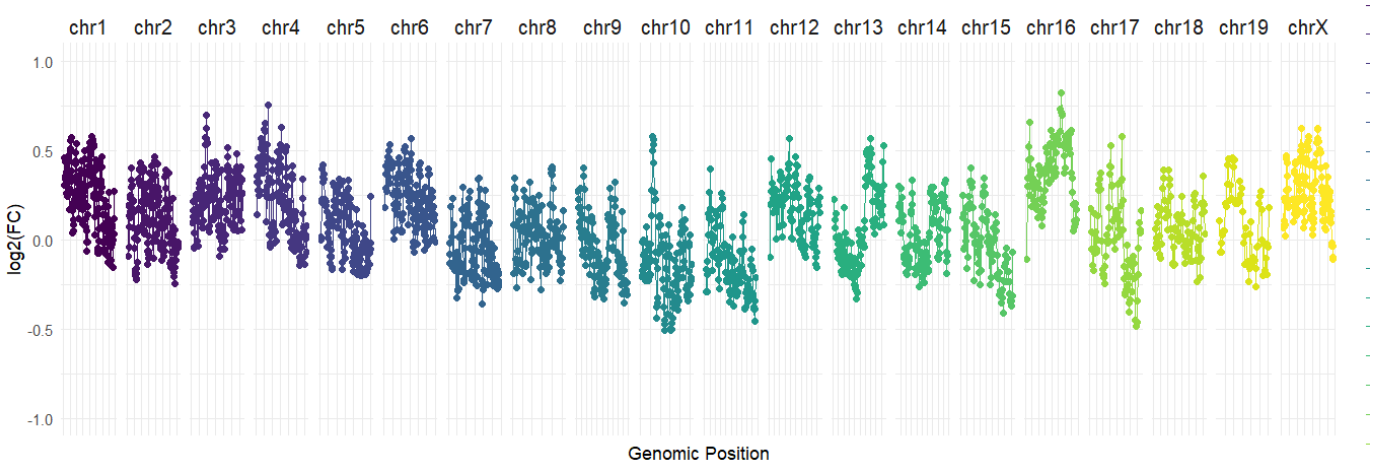
5KO #55



6KO #20



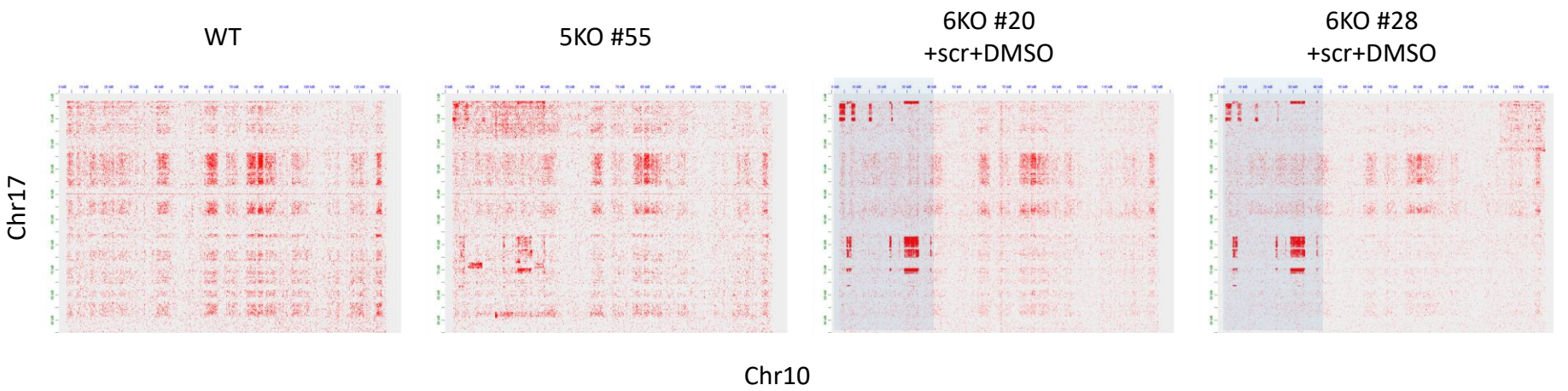
6KO #28



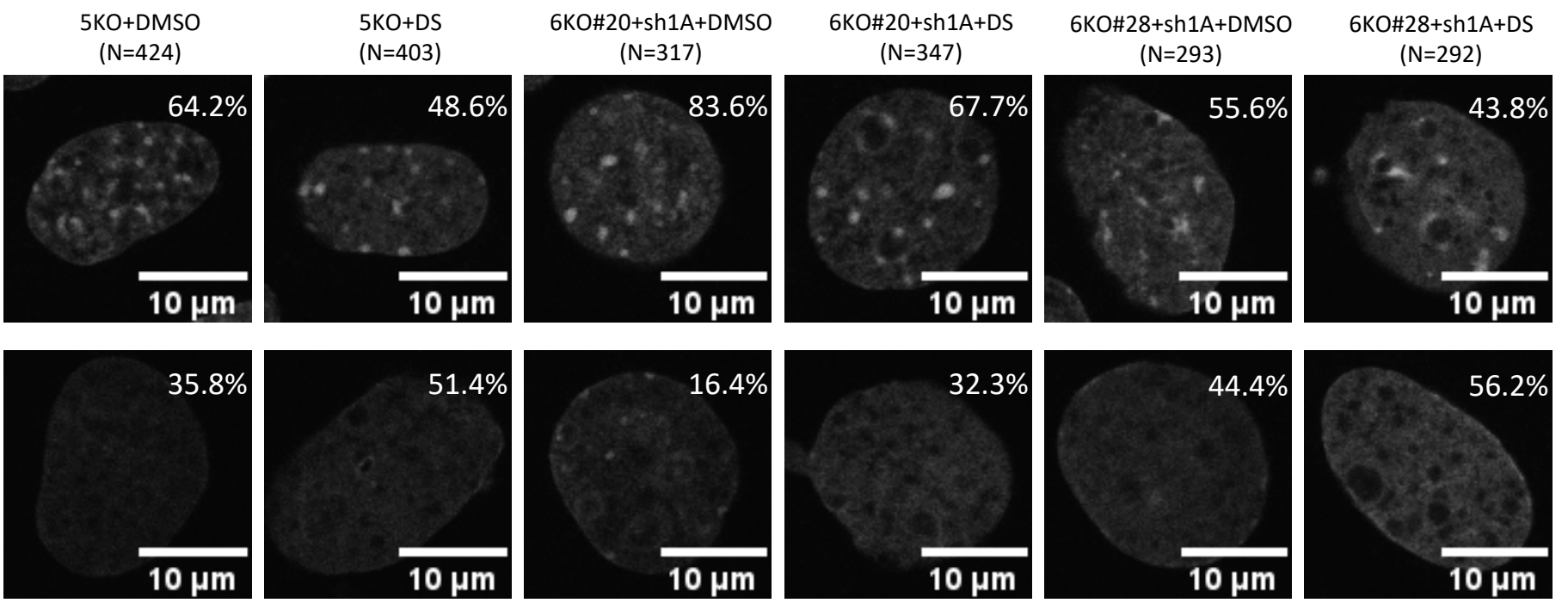
G

chr1	start1	end1	chr2	start2	end2	Sample	Common with
chr10	25000000	40000000	chr17	55000000	76000000	6KO#28	6KO#20
chr10	29000000	35000000	chrX	150000000	165000000	6KO#28	6KO#20
chr1	123000000	140000000	chr11	84000000	94000000	6KO#28	
chr1	132000000	140000000	chr16	5000000	19000000	6KO#28	
chr17	24000000	40000000	chr19	5000000	12000000	6KO#28	WT, 5KO, 6KO#20
chr17	57000000	67000000	chrX	153000000	167000000	6KO#28	6KO#20
chr3	25000000	36000000	chr17	56000000	65000000	6KO#28	6KO#20
chr3	25000000	61000000	chr10	27000000	38000000	6KO#28	6KO#20
chr8	119000000	126000000	chr17	24000000	36000000	6KO#28	WT, 5KO, 6KO#20
chr2	25000000	38000000	chr15	74000000	86000000	6KO#20	WT, 5KO
chr2	25000000	37000000	chr17	24000000	36000000	6KO#20	WT, 5KO
chr3	25000000	71000000	chr10	27000000	38000000	6KO#20	6KO#28
chr3	146000000	157000000	chr13	74000000	84000000	6KO#20	
chr3	25000000	47000000	chr17	56000000	67000000	6KO#20	6KO#28
chr4	132000000	153000000	chr10	75000000	87000000	6KO#20	5KO
chr4	132000000	143000000	chr15	74000000	85000000	6KO#20	5KO
chr8	118000000	126000000	chr15	73000000	85000000	6KO#20	WT, 5KO
chr8	119000000	126000000	chr17	24000000	36000000	6KO#20	WT, 5KO, 6KO#28
chr10	59000000	66000000	chr15	73000000	86000000	6KO#20	
chr10	73000000	101000000	chr15	56000000	88000000	6KO#20	WT
chr10	5000000	16000000	chr17	5000000	16000000	6KO#20	
chr10	23000000	40000000	chr17	54000000	77000000	6KO#20	6KO#28
chr10	29000000	37000000	chrX	128000000	166000000	6KO#20	6KO#28
chr11	94000000	107000000	chr15	73000000	85000000	6KO#20	WT
chr15	73000000	87000000	chr17	23000000	52000000	6KO#20	WT, 5KO
chr15	73000000	86000000	chr19	5000000	12000000	6KO#20	WT, 5KO
chr17	23000000	40000000	chr19	5000000	12000000	6KO#20	WT, 5KO, 6KO#28
chr17	56000000	67000000	chrX	151000000	167000000	6KO#20	6KO#28

H



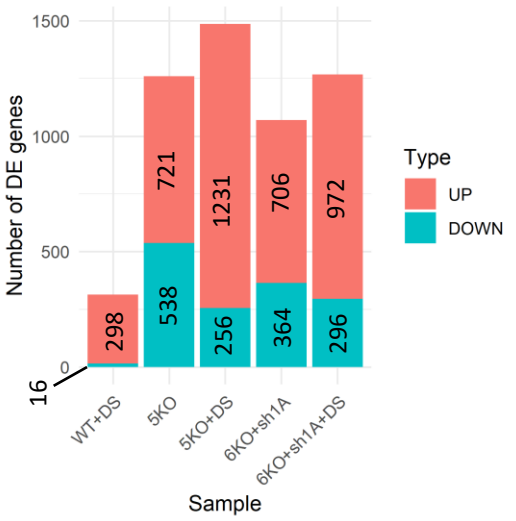
I



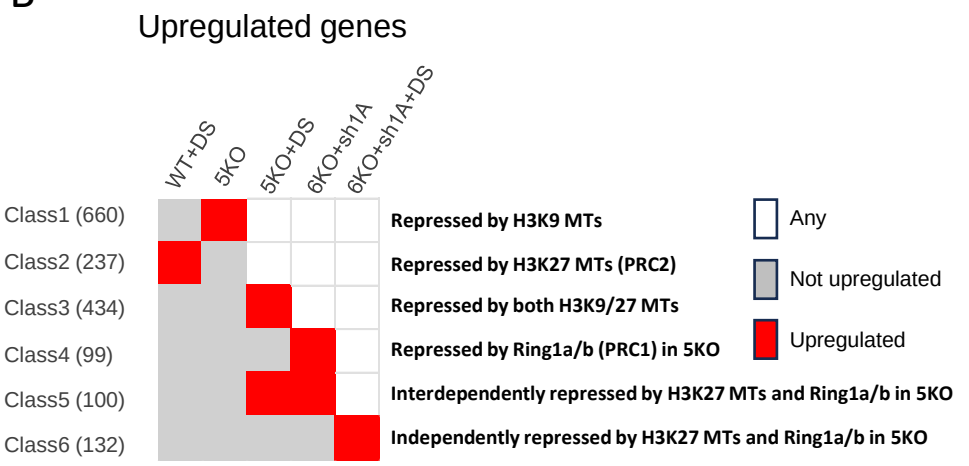
Supplementary Figure S1. Establishment of H3K9 methylation/H3K27me3/uH2A depleted iMEFs. (A) Flow chart illustrating the process of generating 6KO iMEFs. We established two 6KO clones (#20, #28) from 5KO iMEFs. (B) Experimental design of establishment of H3K9 methylation/H3K27me3/uH2A-depleted iMEFs. (C) Western blotting analysis of RING1A and RING1B. *Ring1a*-shRNA treatment efficiently decreased RING1A expression in 6KO iMEFs. (D) Western blotting analysis of uH2A and H3K27me3. Both DS3201 and *Ring1a*-shRNA treatment efficiently decreased uH2A and H3K27me3 in 6KO iMEFs. (E) Relative cell proliferation curve of 5KO and 6KO iMEFs treated with or without DS3201. Cell proliferation was assessed at day 5 and day 7 relative to the starting point. The relative cell number was determined using direct cell counting. The representative data of multiple independent experiments are shown. (F) Karyotype analysis using Hi-C data. The number of mapped reads per 1 Mb was normalized by the total read count, and the log2(FC) relative to WT was calculated and plotted along each chromosome. While no chromosomes exhibited $|\log2(FC)| > 0.5$ across their entire length, smaller variations and region-specific changes were observed, suggesting the presence of chromosomal structural abnormalities in a subset of cells in each sample. (G) List of inter-chromosomal structural variations identified by HiSV. The genomic positions of inter-chromosomal structural variations compared to the reference genome (mm10), and samples with structural variations in the same regions are listed. (H) Representative regions of 6KO iMEF-specific chromosomal rearrangements. Here, chromosomal structural variations between chr10 and chr17 are shown using an interaction matrix derived from Hi-C data. (I) Hoechst-stained cell images. Cells with and without Hoechst-dense foci (chromocenters) were counted. The upper images show representative examples of cells with chromocenters, while the lower images show representative examples of cells without them.

Supplementary Figure 2

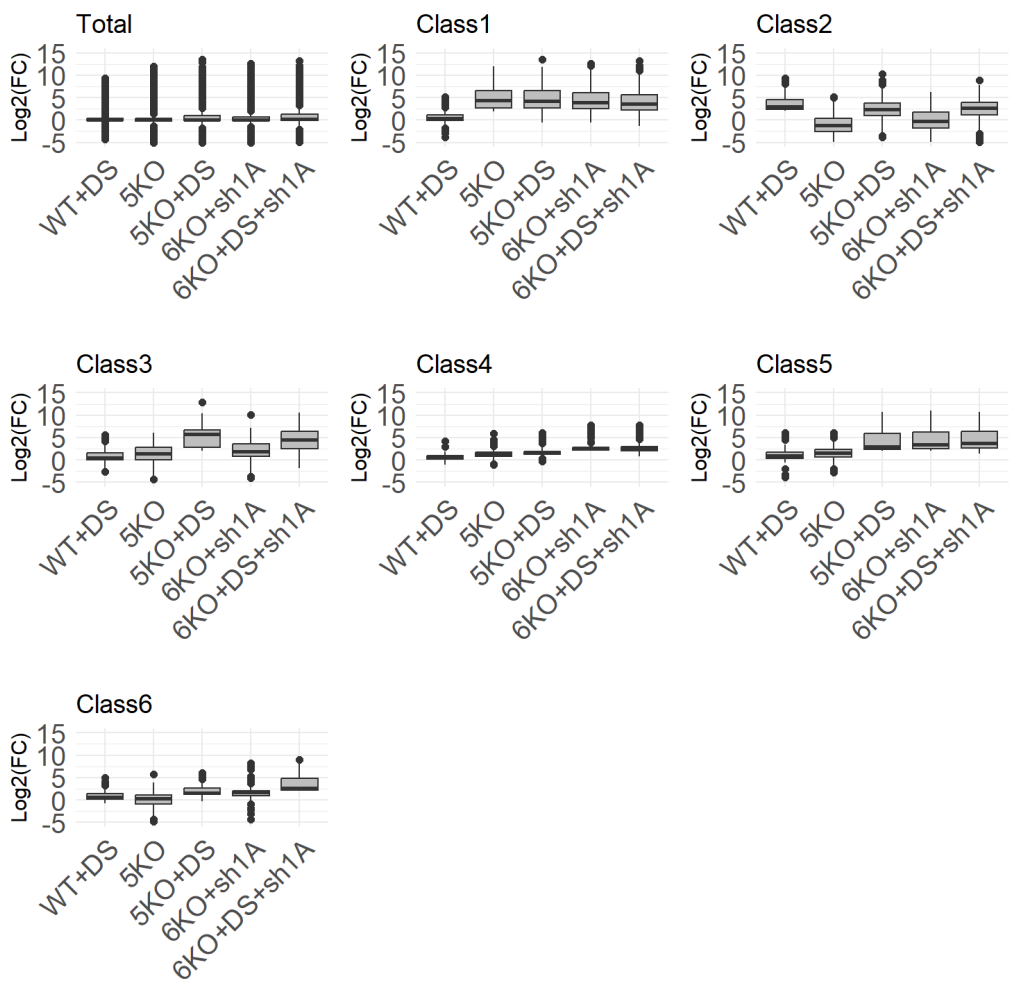
A



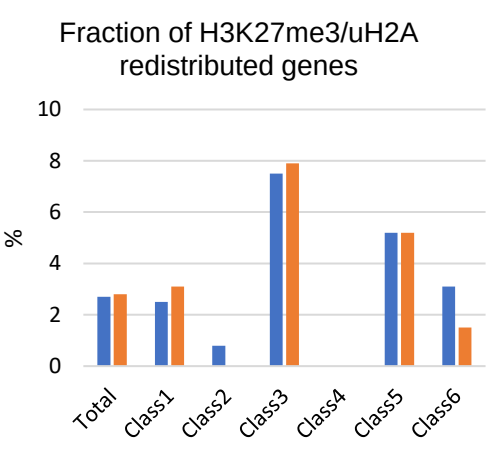
B



C

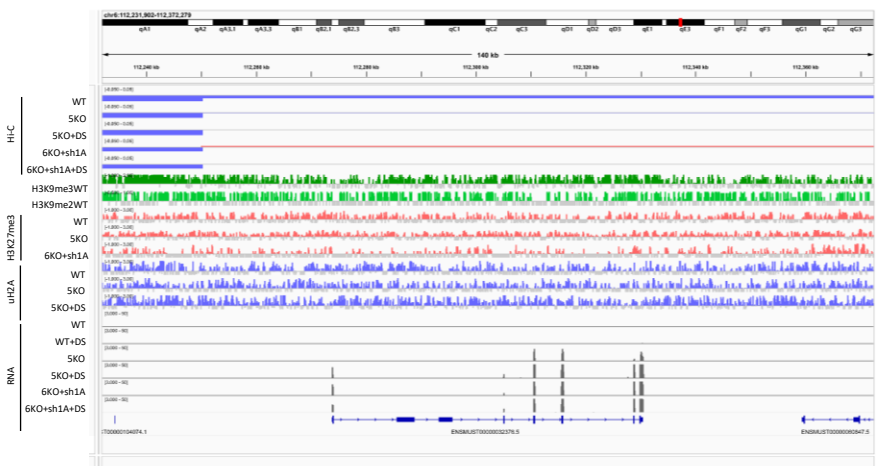


D



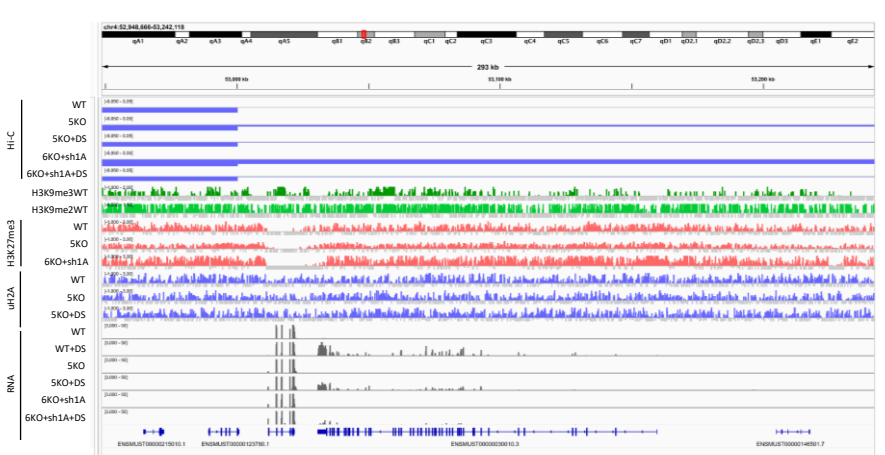
E

Class1 *Lmcd1* loci



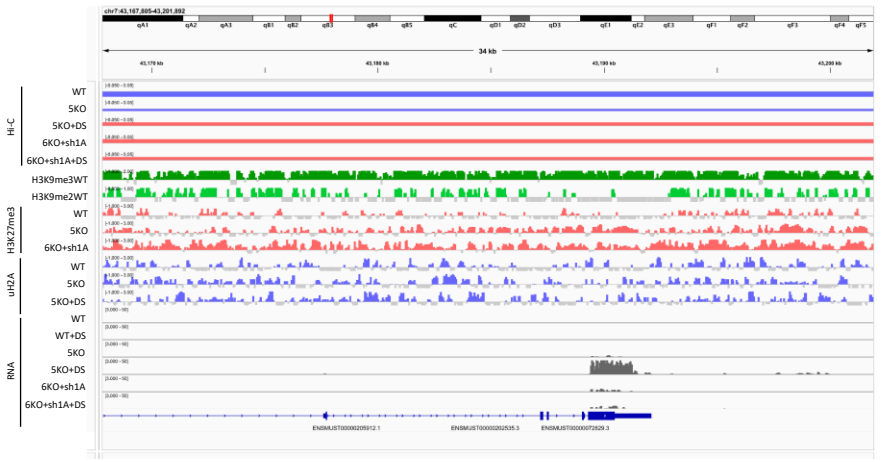
Class4 *Lhx6* loci

Class2 *Abca1* loci

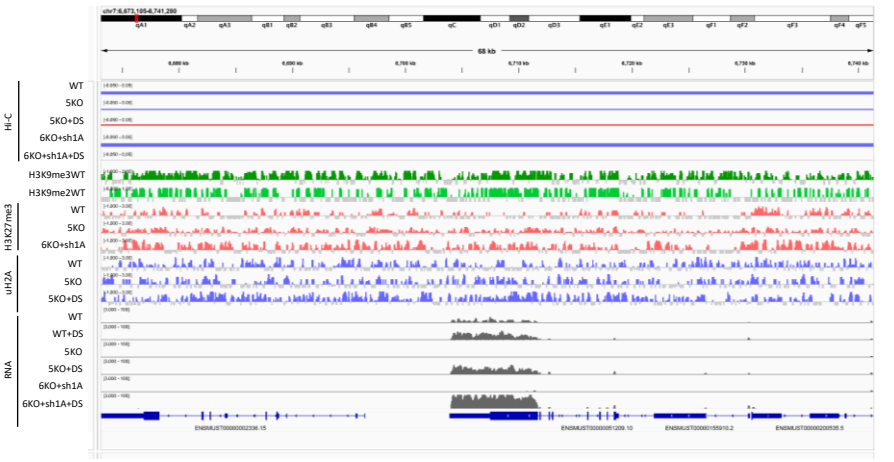


Class5 *Gm2381* loci

Class3 *Zfp936* loci

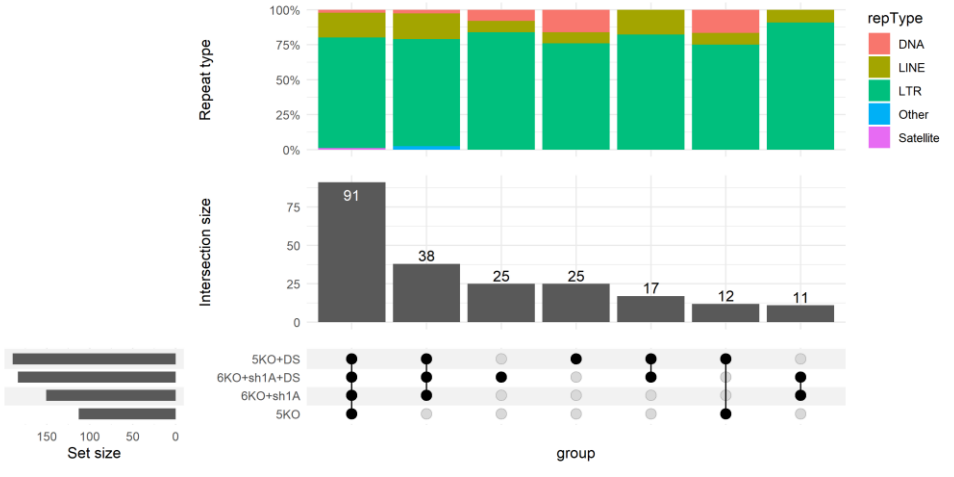


Class6 *Peg3os* loci

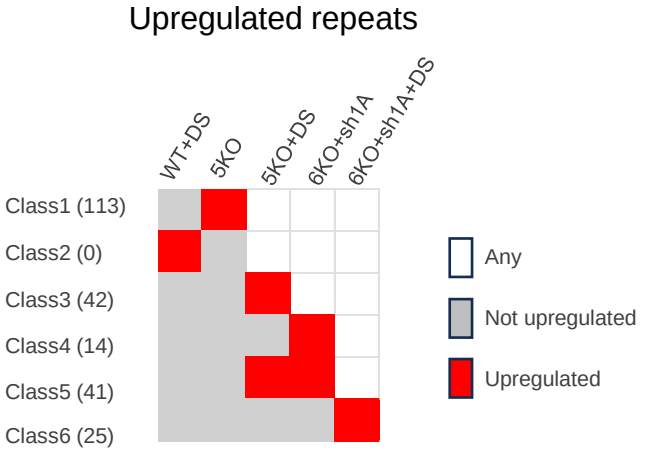


Supplementary Figure S2. Regulation of gene repression by the H3K27me3 and RING1A/B after the loss of H3K9 methylation. (A) Bar plots showing the number of DE genes in each sample. (B) Classification of upregulated genes based on different combinations of upregulated samples. Red, grey, and white represent upregulated, non-upregulated, and any status, respectively. (C) Boxplots showing expression change of upregulated genes. (D) Fraction of H3K27me3/uH2A redistributed genes in upregulated genes. Redistributed genes are those in which H3K9me3 is enriched around the transcription start site in WT ($\log_2$(ChIP/Input) > 0), while H3K27me3 and uH2A are not enriched ($\log_2$(ChIP/Input) < 0), but in 5KO, H3K27me3 and uH2A become enriched. (E) Representative region of upregulated genes in each class.

A



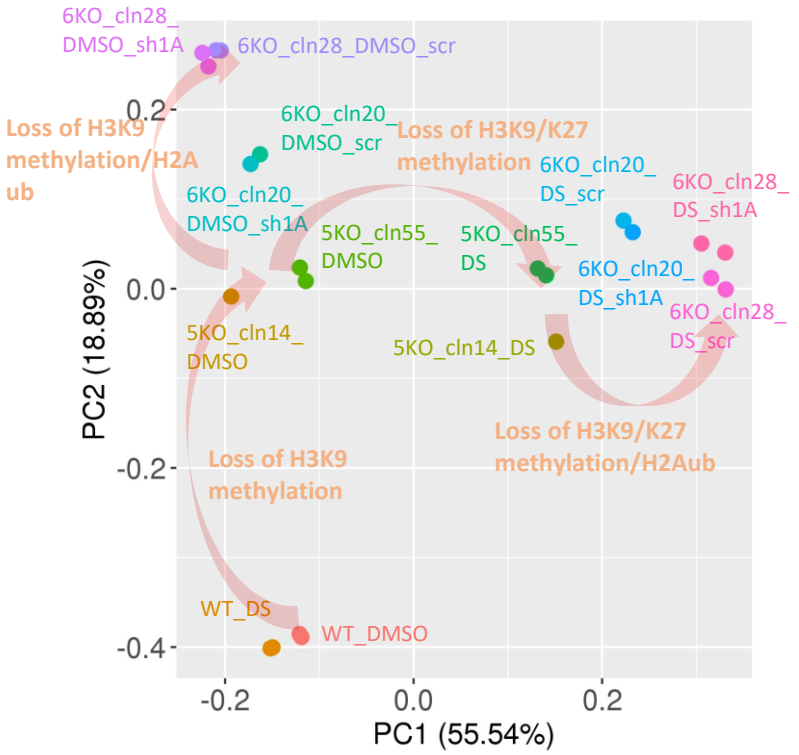
B



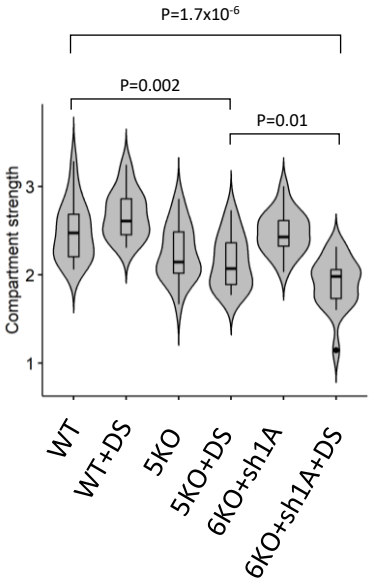
Supplementary Figure S3. Regulation of transposon repression by the H3K27me3 and RING1A/B after the loss of H3K9 methylation. (A) Upset plot of upregulated transposons. Bar plots show the fraction of repeat type of each group. (B) Classification of upregulated genes based on different combinations of upregulated samples. Red, grey, and white represent upregulated, non-upregulated, and any status, respectively.

Supplementary Figure 4

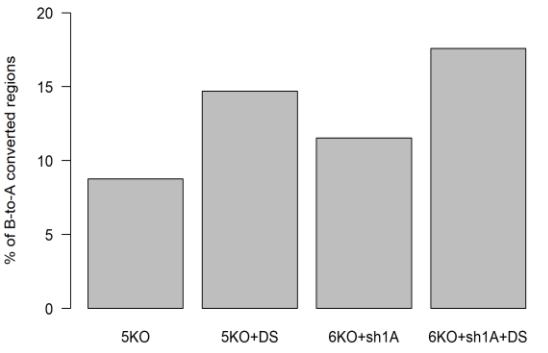
A



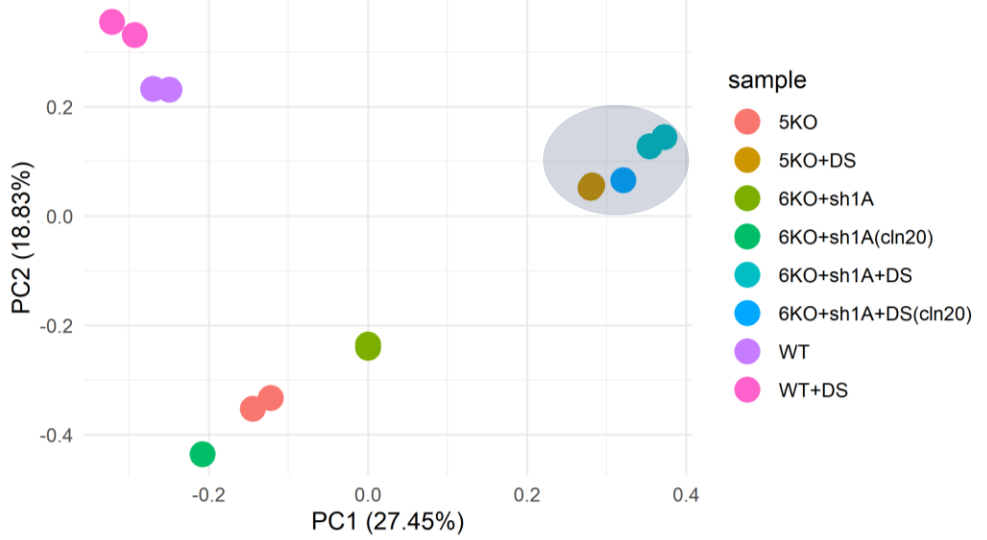
B



C



D



Supplementary Figure S4. H3K27me3 and RING1A/B maintain A/B compartment segregation after the loss of H3K9 methylation. (A) PCA plot of Hi-C PC1 values of 250-kb bin. (B) Violin plots of compartment strength. P-values were calculated by Tukey's test. (C) Fraction of B-to-A converted 250-kb bin. (D) PCA plot of insulation score of 40-kb bin.