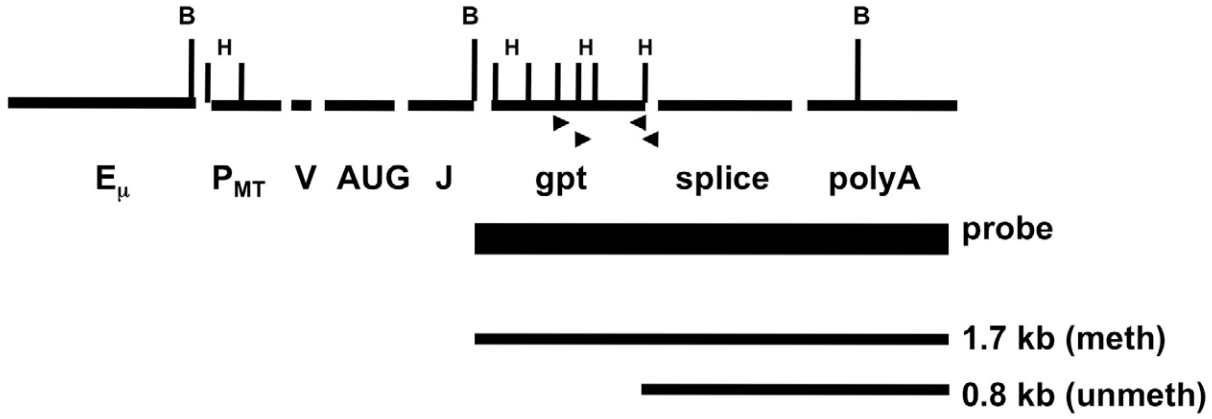

Fig. S1: HRD transgene. HRD construct introduced into mouse ESCs. Mice with the HRD transgene were obtained from established ESC lines. The HRD transgenic mice (line #342) mainly used in this report had 6 copies of the HRD transgene (Engler et al. 1991). Eμ, Ig heavy chain enhancer; PMT, metallothionein-1 promoter; V, J, Ig recombinase recognition sequences; AUG, rat initiation codon and surrounding sequences; gpt, *E.coli gpt* (guanine phosphoribosyl transferase) coding sequence; splice polyA are from SV40. *HpaII* (H) and *Bam*HI (B) restriction sites, probe used for Southern blot analysis and the expected band sizes for methylated (meth) and unmethylated (unmeth) regions are shown. Nested PCR primers used for the bisulfite analysis of the 2nd half of the *gpt* region are also indicated.

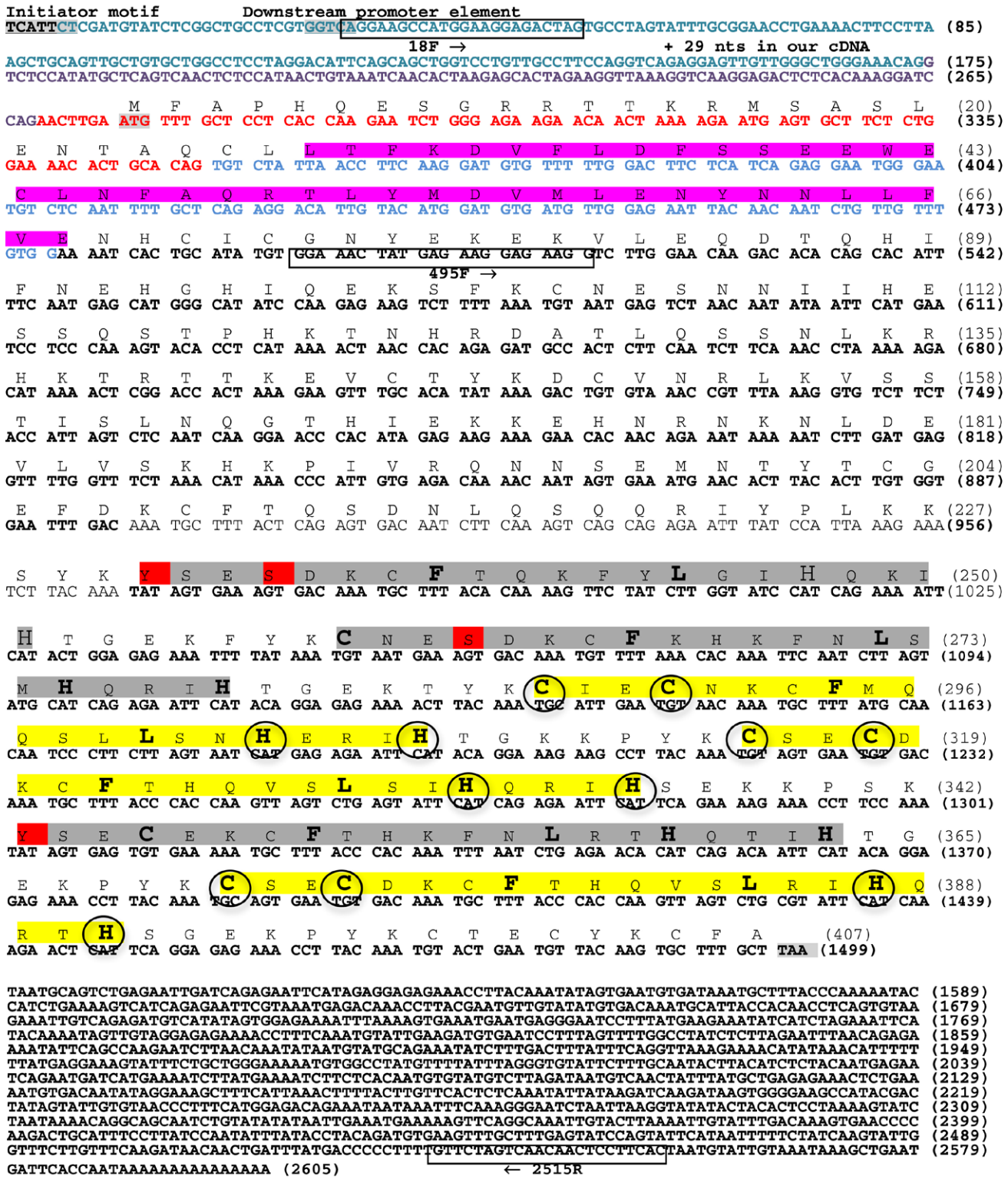
Initiator motif
Downstream promoter element
18F →
+ 29 nts in our cDNA
495F →
← 2515R
exon 1
exon 2
exon 3
exon 4
exon 5
KRAB domain
Non functional zinc fingers
Functional zinc fingers
Amino acid change that makes a potential zinc finger non-functional

Fig. S2: cDNA and amino acid sequence of *Ssm1b*. The *Ssm1b* cDNA consists of five exons that are marked in different colors. The corresponding amino acids are noted above their respective codons. The additional 29 nts that we obtained in our PCR amplification (but is not observed in NM_145078) is underlined. The numbers in parentheses are the nucleotide/amino acid number. The initiator element and the downstream promoter enhancer are also underlined. In the amino acid sequence the KRAB domain and the zinc fingers are demarcated with different colors. The functional zinc fingers are colored yellow while the non-functional ones are in gray. The essential cysteines and histidines in a zinc finger are circled. The amino acid change that makes a zinc finger non-functional is colored red. The primers 18F, 495F and 2515R used to analyze the Ssm1 DNA and cDNA are boxed.

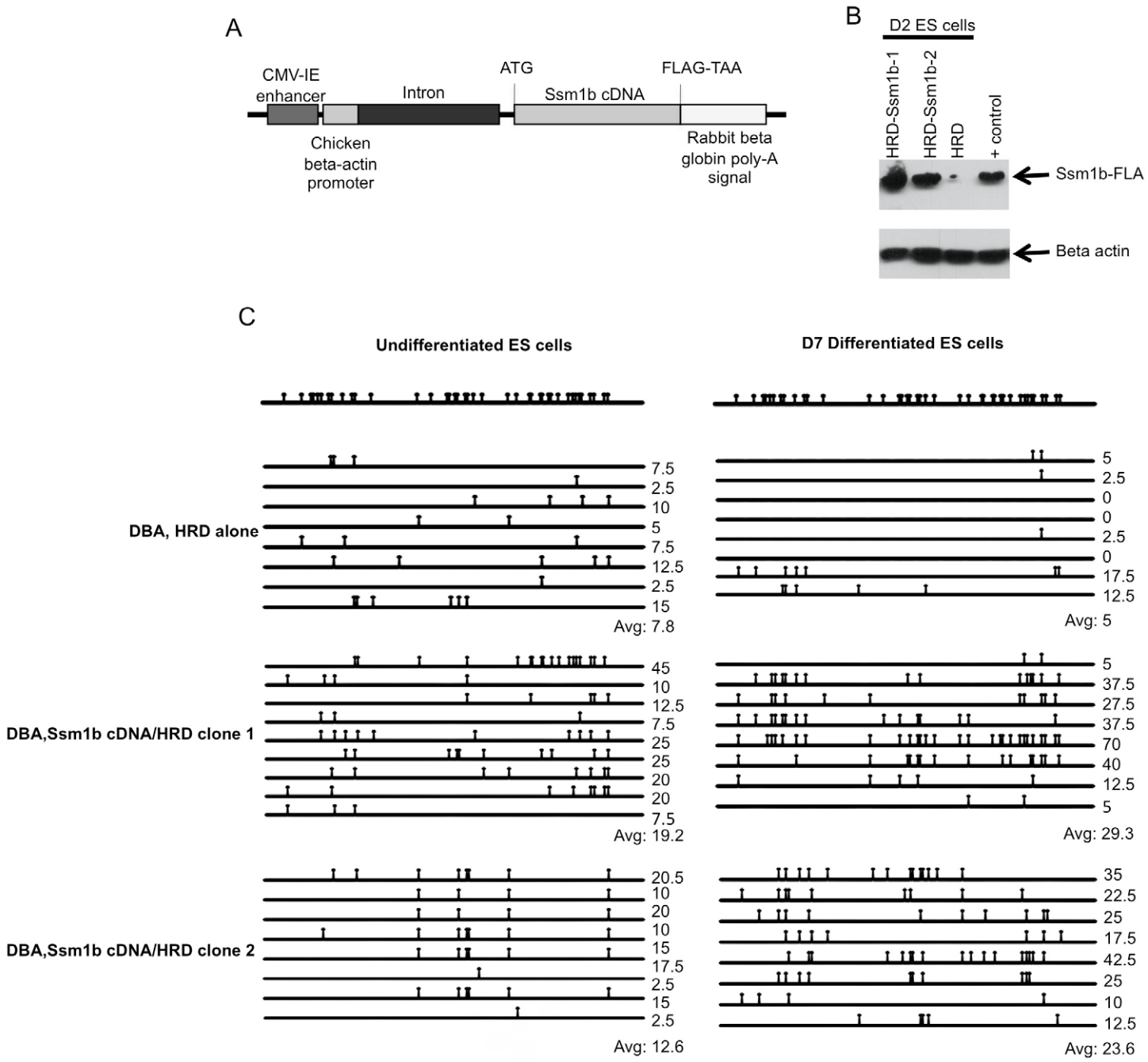

Fig. S3: *HRD* gains methylation upon differentiation of D2 ESCs expressing *Ssm1b* cDNA. (A) *Ssm1b*-FLAG cDNA construct that was introduced into D2 (non-methylating strain) ESCs. (B) Western blot showing expression of Ssm1b-FLAG protein in D2 ESCs transfected with Ssm1b-FLAG cDNA construct. + control: ESCs protein lysate from a previous experiment expressing Ssm1b-Flag protein. (C) Bisulfite analysis: Increase in *HRD* methylation in two independent clones of D2 ESCs expressing the *Ssm1b-FLAG*-cDNA transgene.

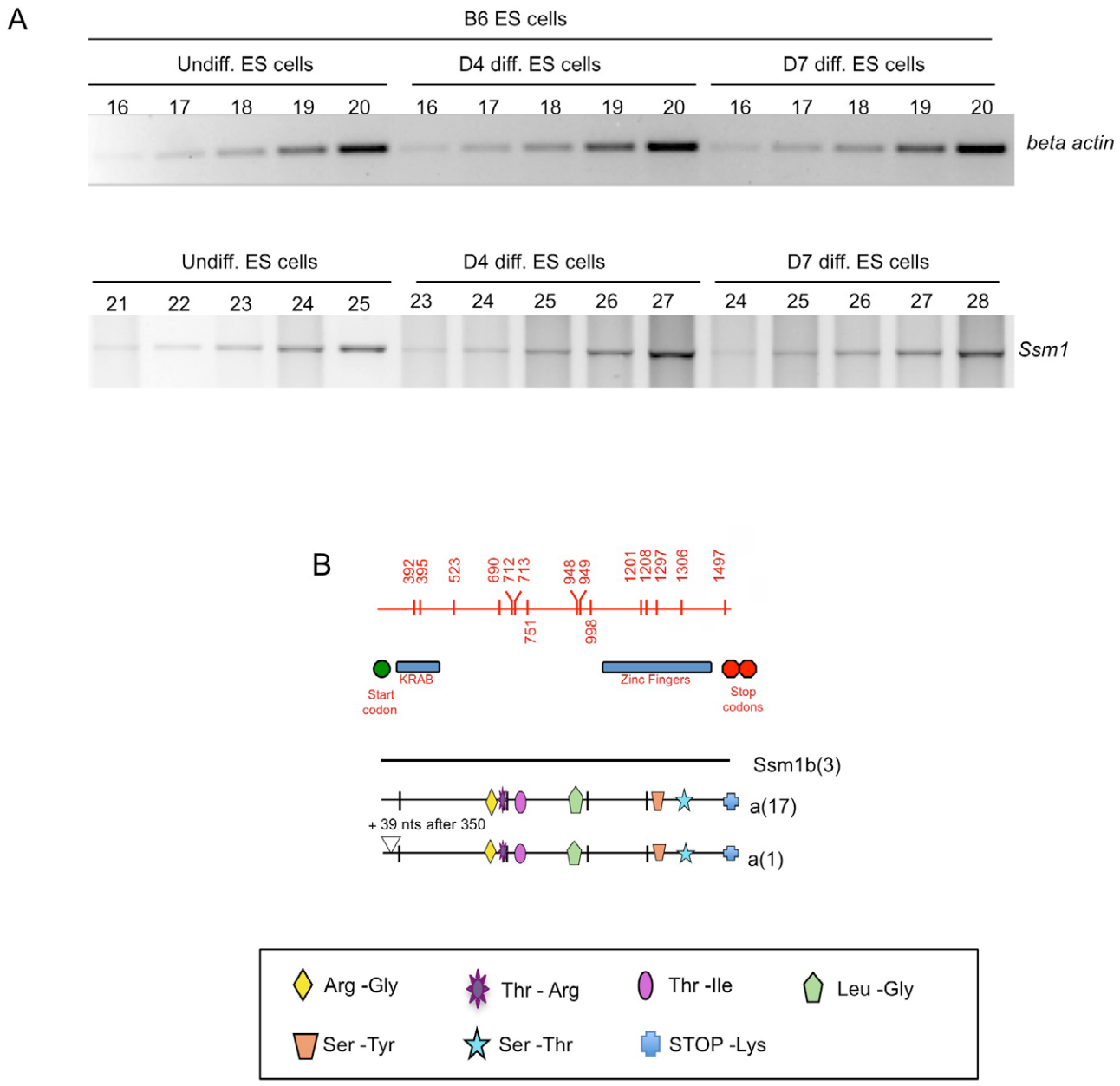

Fig. S4: Decrease in *Ssm1* expression upon ESC differentiation. (A) RT-PCR of *beta actin* (top panel) and *Ssm1*(bottom panel) in B6 undifferentiated, day 4 (D4) differentiated and D7 differentiated ESCs. The number of PCR cycles are depicted as numbers above the gels. Ssm1 expression in D7 differentiated cells falls to 12.5% of its expression level in undifferentiated cells. (B) Expression of *Ssm1* in B6 D4 differentiated ESCs. The *Ssm1* cDNA PCR amplified product amplified [using primers 18F and 2515R (Table S1)] was cloned and sequenced. Each horizontal line represents a sequence from 1, 3 or 17 bacterial clones. The lines labeled *Ssm1b* represent the sequence that matches the genomic sequence of *Ssm1b* and hence is the *Ssm1b* cDNA. The lines marked a and b represent the other *Ssm1b*-like sequences. The numbers in parentheses indicate the number of clones for each sequence. SNPs causing amino acid changes are marked with a symbol and the actual amino acid changes are depicted below.

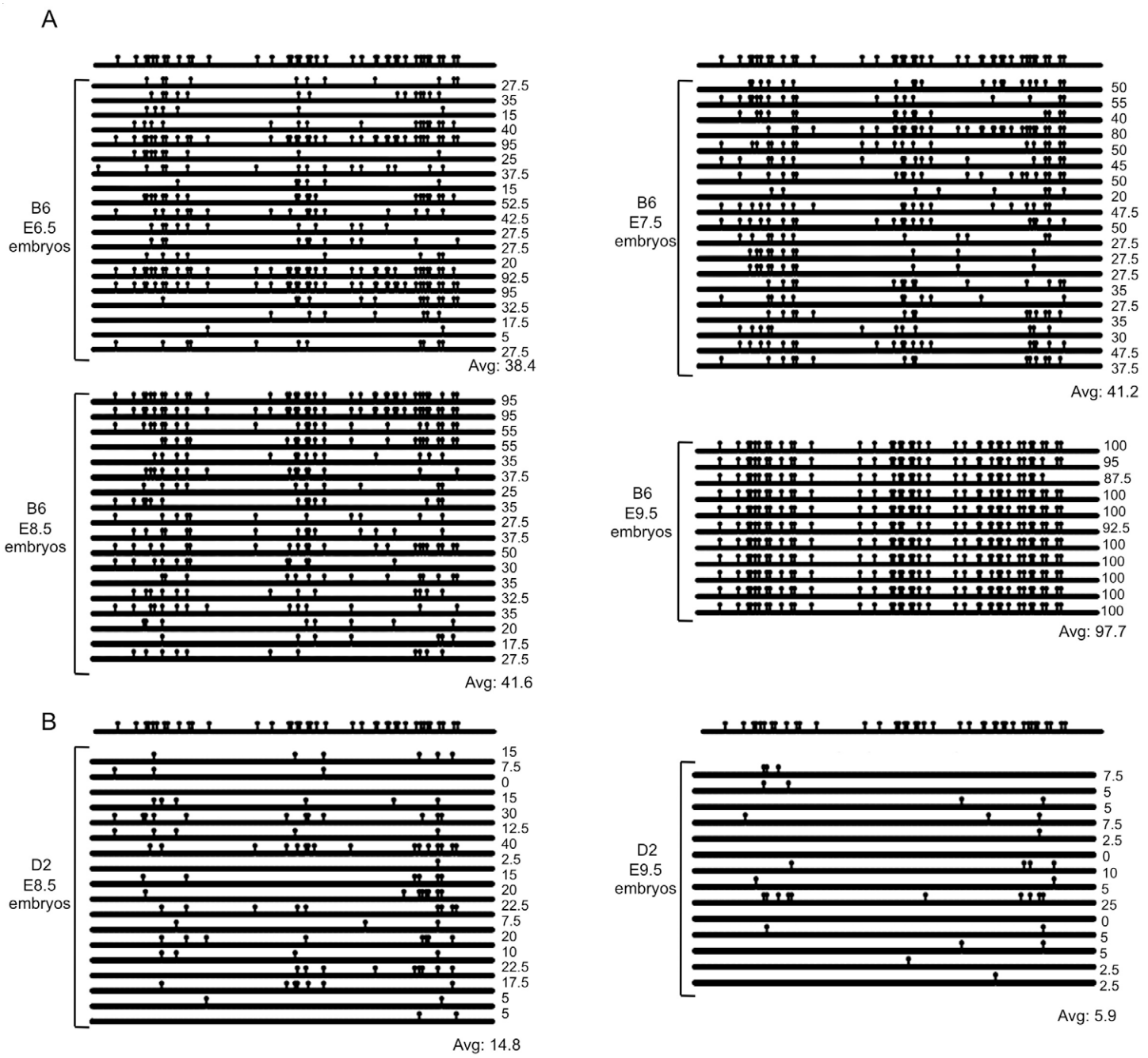

Fig. S5: HRD-*gpt* methylation during early mouse development. (A) Bisulfite analysis showing gain in HRD methylation from E6.5 to E9.5 (B6 x D2)F1 mouse embryos. All CpG dinucleotides present in the analyzed sequence are shown above the data. Numbers by each horizontal line indicate the percentage of CpGs methylated per sequence; Avg = average % of CpG methylation. (B) Bisulfite analysis showing low HRD methylation in E8.5 and E9.5 D2 mouse embryos.

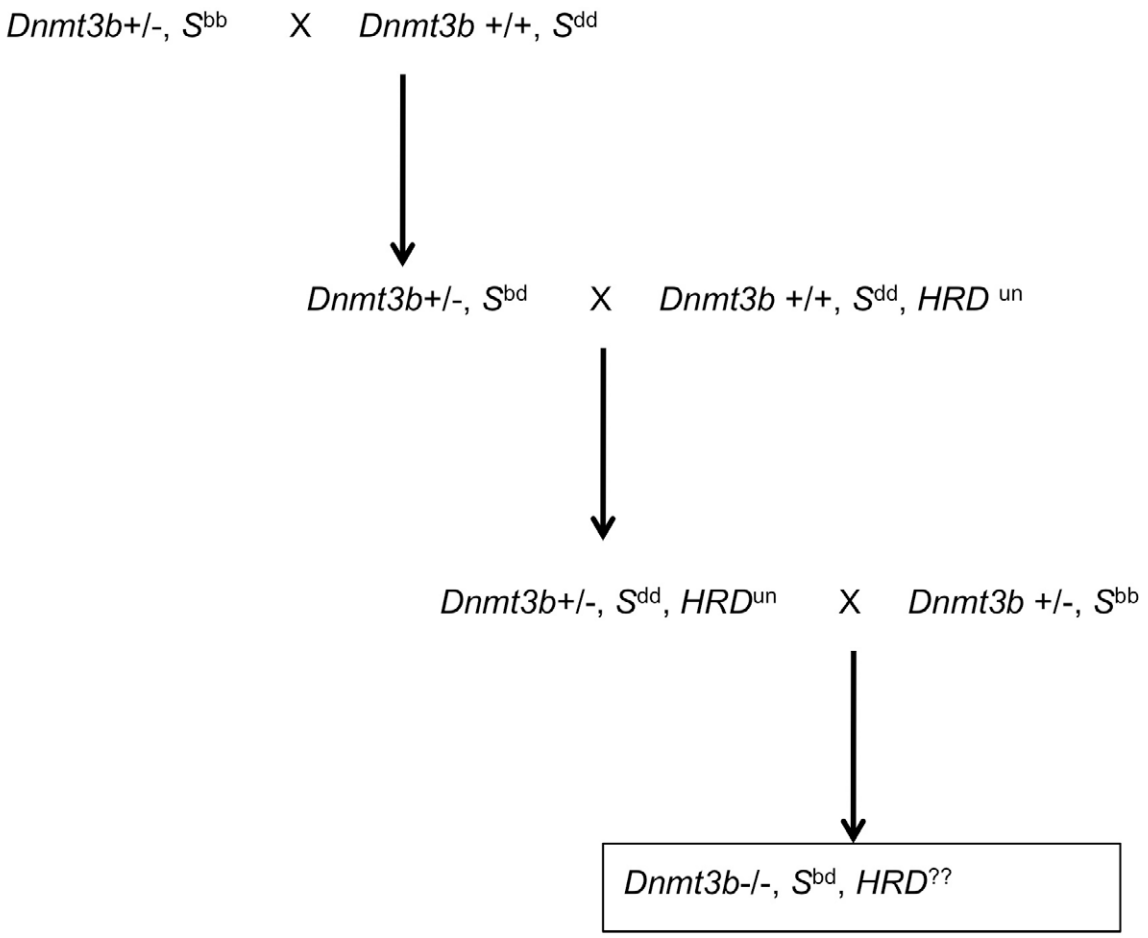

Fig S6: Breeding scheme used to obtain *Dnmt3b* knockout embryos with *Ssm1b* expression. *Dnmt3b*+/+ = *Dnmt3b* wildtype mouse, *Dnmt3b*+/- = *Dnmt3b* heterozygous mouse, *Dnmt3b* -/- = *Dnmt3b* homozygous knockout mouse; S^{bb} = *Ssm1b* homozygous mouse, S^{bd} = *Ssm1b/Ssm1d* heterozygous mouse, S^{dd} = *Ssm1d* homozygous mouse (and therefore lacking *Ssm1b* expression). HRD^{un} = unmethylated HRD, $HRD^{??}$ = HRD methylation status to be analyzed.

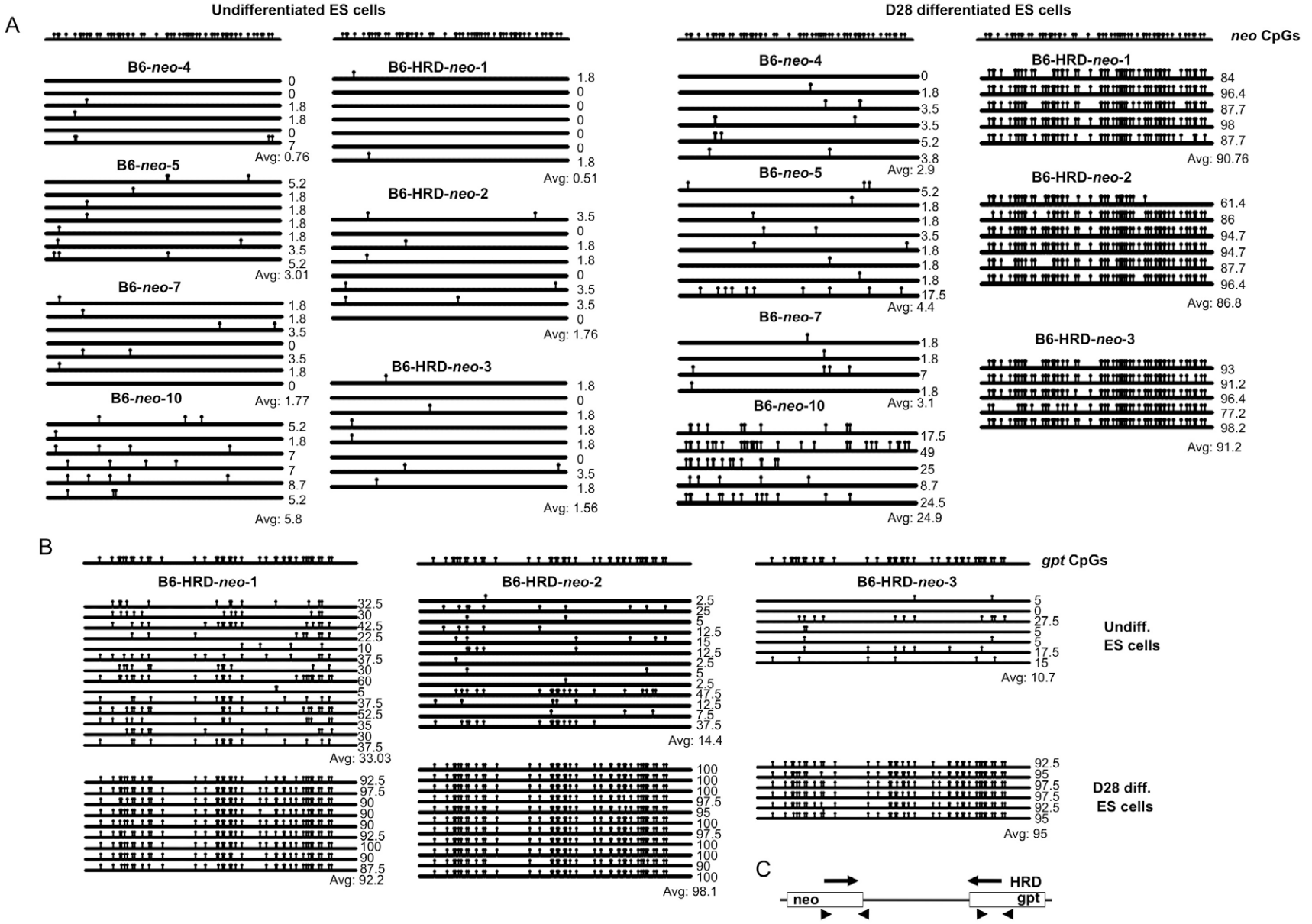

Fig. S7: Specific methylation of *neo* gene in HRD-*neo* but not of *neo* gene alone upon differentiation. (A) Bisulfite analysis showing lack of methylation in undifferentiated B6 ESCs of *neo* when alone (*B6-neo*) or when coupled to HRD (HRD-*neo,* map shown in C) (the two left panels). Upon differentiation to D28, *neo* remains unmethylated and HRD-*neo* shows complete methylation (the two right panels). All CpG dinucleotides present in the analyzed *neo* sequence are shown above the data. B6-*neo*-4, B6-*neo*-5, B6-*neo*-7 and B6-*neo*-10 are the four independent ESC lines transfected with *neo* alone, while B6-HRD-*neo*-1, B6-HRD-*neo*-2, B6-HRD-*neo*-3 are the ESC lines transfected with HRD-*neo*. (B) Bisulfite analysis showing partial methylation of *gpt* in undifferentiated ESCs (Undiff. ESCs, upper panel) but increased *gpt* methylation in HRD-*neo* cells differentiated to D28 (D28 diff ESCs, lower panel). All CpG dinucleotides present in the analyzed *gpt* sequence are shown above the data. (C) HRD-*neo* construct introduced into B6 ESCs. Primers used to analyze the bisulfite-treated *neo* and *gpt* regions are indicated below the construct.

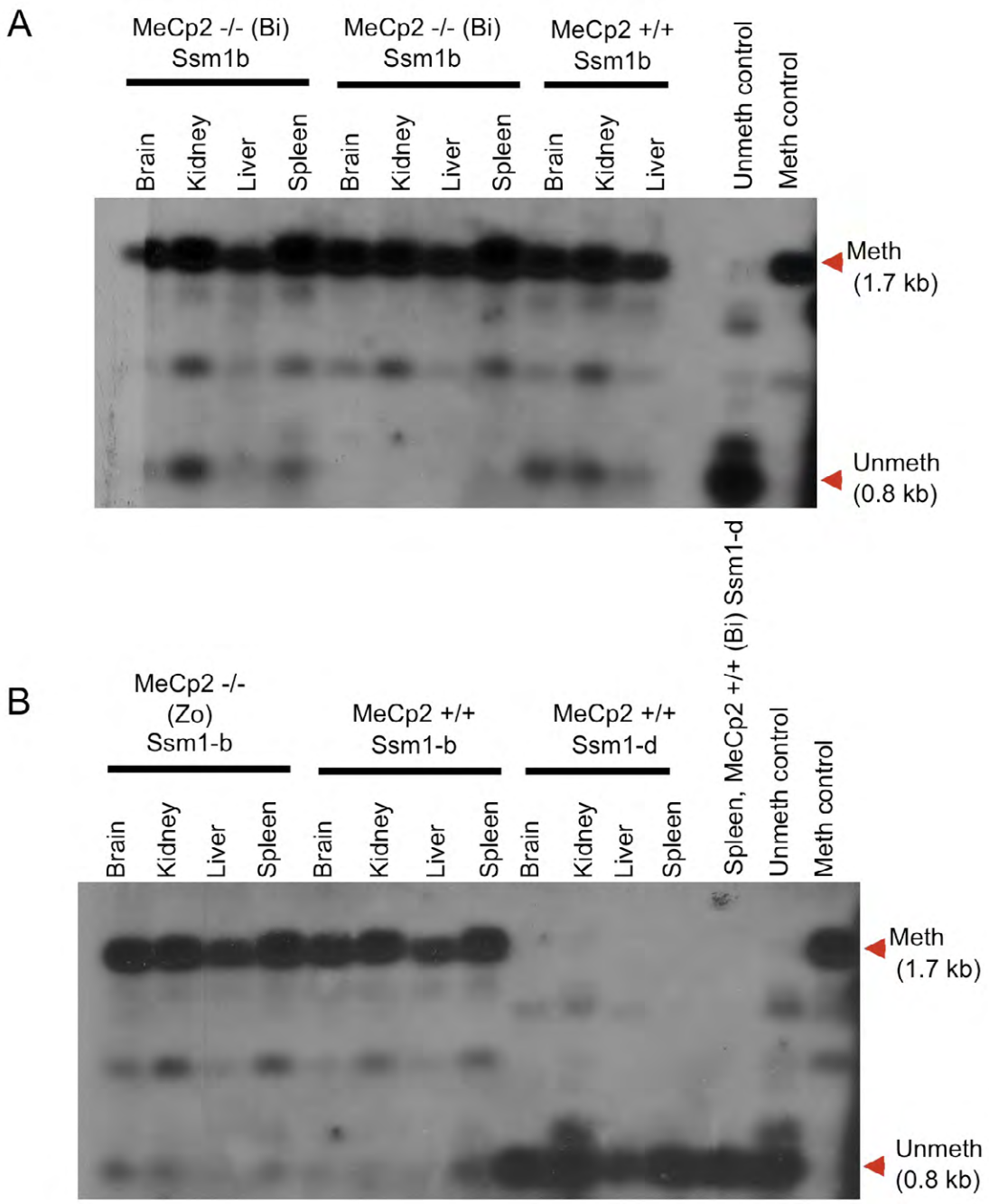

Fig. S8: Role of Mecp2 in HRD/*gpt* methylation. Southern blots probed with gpt sequence showing that HRD stays methylated in various organs from mice lacking Mecp2 expression (*Mecp2*-/-) as in control littermates (*Mecp2*+/+). (A) Mice with complete inactivation of *MeCp2* (Bi); (B) Mice with truncation of *MeCp2* within exon 4 (Zo). Meth: uncut (methylated) band, Unmeth: cut (unmethylated) band.

Table S1. Primers used in this study

Gene	Primers	Product size	Location	Cycling conditions
BAC DNA	pBACe3.6F1 ATTTGGCGGTGTTGATACAGCGGG pBACe3.6R1 GGGGTGATAGTGTTGAGAAGACCTC	417 bp	In the BAC vector pBACe3.6	(94°C for 30 sec, 57°C for 30 sec, 72° C for 45 sec) for 30 cycles
Ssm1b cDNA construct	3622F GTCTCAATCAAGGAACCCACA 4448R GTGGTATTTGTGAGCCAGGGCATT	827 bp	Part of the linker region, Zn Finger domain, 3'UTR and part of the rabbit beta-globin polyA region in the construct	(94°C for 30 sec, 57°C for 30 sec, 72° C for 45 sec) for 35 cycles
Endogenous Ssm1 cDNA expression	18F CAGGAAGCCATGGAAGGAGACTAG 2515R GTGAAGGAGTTGTTGACTAGAAC	2.5 kb	Most of the 5'UTR, KRAB domain, linker region, Zn Finger domain and most of the 3'UTR of Ssm1b	(94°C for 30 sec, 56°C for 30 sec, 72° C for 2.5 min +2 sec/cycle) for 20 cycles followed by (94°C for 30 sec, 56°C for 30 sec, 72° C for 5 min for 15 cycles.

Ssm1b DNA	495F GGAAACTATGAGAAGGAGAAGG 2515R GTGAAGGAGTTGTTGACTAGAAC	2 kb	Linker region, Zn Finger region and most of the 3'UTR of Ssm1b	(94°C for 30 sec, 60°C for 30 sec, 72° C for 2 min) for 30 cycles
Dnmt3b	Common forward primer TCACAGGGTACTTGGTGCTCAAGGA Wild-type reverse primer TACCTCCAACTGCCCTGTTTGCACT KO Reverse primer ACACTCCAACCTCCGCAAACTCCTA	+/+ 300 bp +/- 300 bp and 223 bp -/- 223 bp	Li *et al* 1992	(94°C for 10 sec, 60°C for 30 sec, 72° C for 30 sec) for 30 cycles
HRD	PMTF CGCTCATGTGAAGTGTCCCAG GPTR CCTCACTTACTCCGTAGCTCC	450 bp	Region from the *HRD* promoter to part of the *gpt* region in *HRD*	(94°C for 30 sec, 58°C for 30 sec, 72° C for 45 sec) for 30 cycles
Mecp2 (Bi)	Common primer oIMR 1436 GGT AAA GAC CCA TGT GAC CC Wild-type primer oIMR GGC TTG CCA CAT GAC AA KO primer oIMR 1437 TCC ACC TAG CCT GCC TGT AC	+/+ 416 bp +/- 400 bp and 416 bp -/- 400 bp	Guy *et al.* 2001	(94°C for 45 sec, 60°C for 1 min, 72° C for 1 min) for 35 cycles

Mecp2 (Zo)	Common primer oIMR 3912 AAC GGG GTA GAA AGC CTG Wild-type primer oIMR 3913 TGA TGG GGT CCT CAG AGC KO primer oIMR 3914 ATG CTC CAG ACT GCC TTG	+/+ 396bp +/- 318bp and 396bp -/- 318bp	Shahbazian *et al*. 2002	(94°C for 45 sec, 62°C for 45 sec, 72° C for 45 sec) for 35 cycles
Bisulfite-treated *gpt*	GPT F7 ATTGATGATTTGGTGGATATT SV40B11 CACCACTACTCCCATTCATC GPT F10 TGTATTTTGTTATTATTTTT SV40B10 CAAAACCCACTCATAAATCC	518 bp (nested PCR product)	2nd half of the *gpt* region in *HRD*	(94°C for 1 min, 56°C for 2 min, 72° C for 2 min) for 35 cycles
Bisulfite-treated *neo*	Neo-F732 GGTTATTGAATAAGATGGATTGTA Neo-R1430 CCAAACTCTTCAACAATATCAC	699 bp	Neomycin-resistant gene in *HRD* and in *neo*	(94°C for 1 min, 58°C for 1 min, 72° C for 2 min) for 30 cycles.

Table S2. Comparison of *Ssm1b* expression and HRD methylation during development.

	E6.5		E7.5		Early E8.5		Late E8.5		E9.5		Adult tissues		EET		ESCs		Differentiated ESCs (d 28)	
Mouse strain	**B6**	**D2**	**B6**	**D2**	**B6**	**D2**	**B6**	**D2**	**B6**	**D2**	**B6**	**D2**	**B6**	**D2**	**B6**	**D2**	**B6**	**D2**
Ssm1b expr.	Yes	NA	Yes	NA	Yes	NA	Yes	NA	No	NA	No	No	NA	NA	Yes	No	NA	NA
Ssm1-like expr.	Yes	NA	Yes	NA	Yes	NA	Yes	NA	Yes	NA	Yes	No	NA	NA	Yes	Yes	NA	NA
HRD meth	2+	0	2+	0	2+	NA	2+	0	3+	0	3+	0	*	*	1+	0	3+	0

The expression of Ssm1b and other Ssm1 family members was analyzed in B6 and D2 embryos (E6.5-E9.5), adult tissues (heart, blood), gametes, extra-embryonic tissues (EET) from various stages of early development, ESCs and day 28 differentiated ESCs. Expression (expr.) of Ssm1b was seen up to early E8.5 in B6 embryos and in undifferentiated ESCs. Ssm1b was never expressed at any stage in D2. The corresponding gain in HRD methylation is indicated by number with 0 depicting methylation no higher than 14.8% and 3+ depicting complete methylation based on percentage of CpG methylated in bisulfite assay. For EET, Southern blots showed intermediate methylation. *, strain independent. NA, not analyzed.