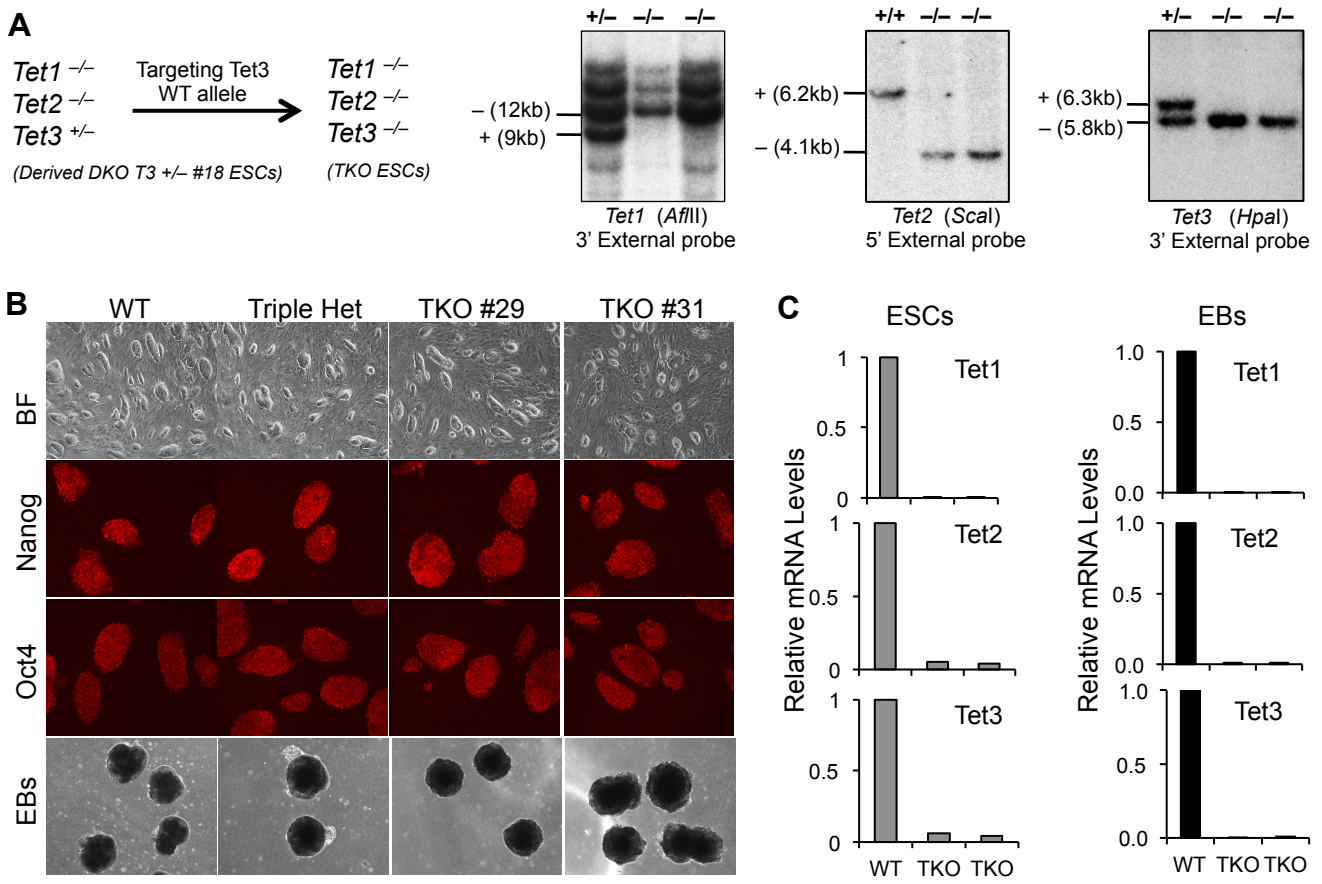

Figure S1: Generation of Tet1/2/3 triple knockout (TKO) mouse embryonic stem cells (Related to Figure 1). (A) Schematic of the targeting step to generate Tet1/2/3 triple knockout mouse ES cells and Southern blot for genotype confirmation of derived lines. (B) Bright-field images of ESCs and EBs stained with antibodies against the pluripotency markers Oct4 and Nanog in ESCs of the indicated genotypes. (C) RT-qPCR for Tet1, Tet2, and Tet3 in ESCs and embryoid bodies of the indicated genotypes showing TKO ESCs and EBs are depleted of Tet1, Tet2 and Tet3 mRNA. Data are normalized to Gapdh.

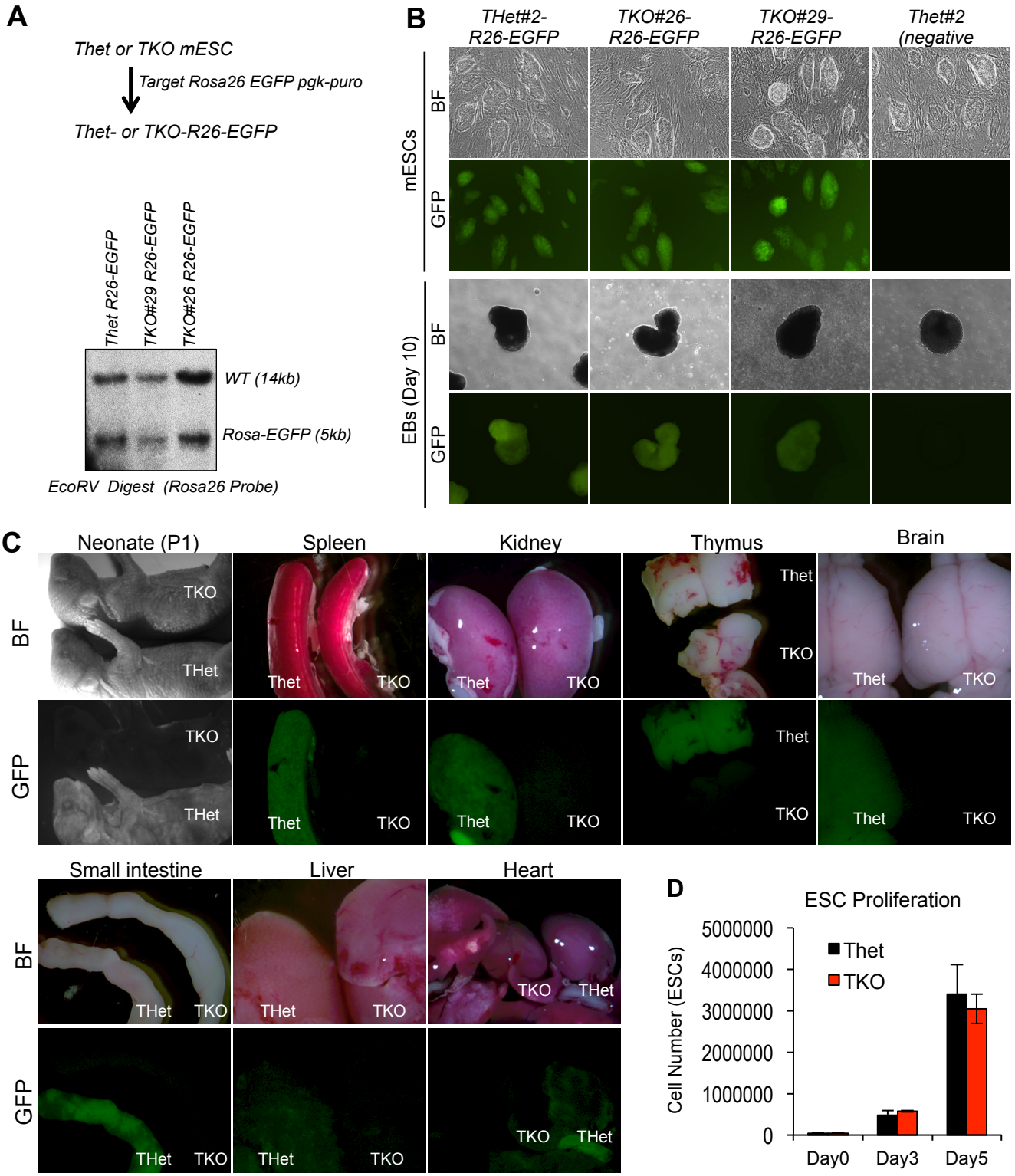

Figure S2: Generation of Rosa26-EGFP-pgk-puro targeted TKO mESCs for use in chimera assays (Related to Figure 2). (A) Southern blot confirming properly targeted Rosa26-EGFP clones of indicated ESCs. (B) Bright field and fluorescence images of targeted ESCs and EBs derived from these cells confirming ubiquitous EGFP expression. (C) Gross images of organs from chimeric mice: Absence of EGFP signal in various organs of 4-week-old mice derived from blastocysts injected with TKO-Rosa26-EGFP ESCs. THet-Rosa26-EGFP ESCs are used as controls. (D) Proliferation rate of TKO ESCs assessed by cell count over several days in culture. Error bars indicate standard deviation.

Figure S3

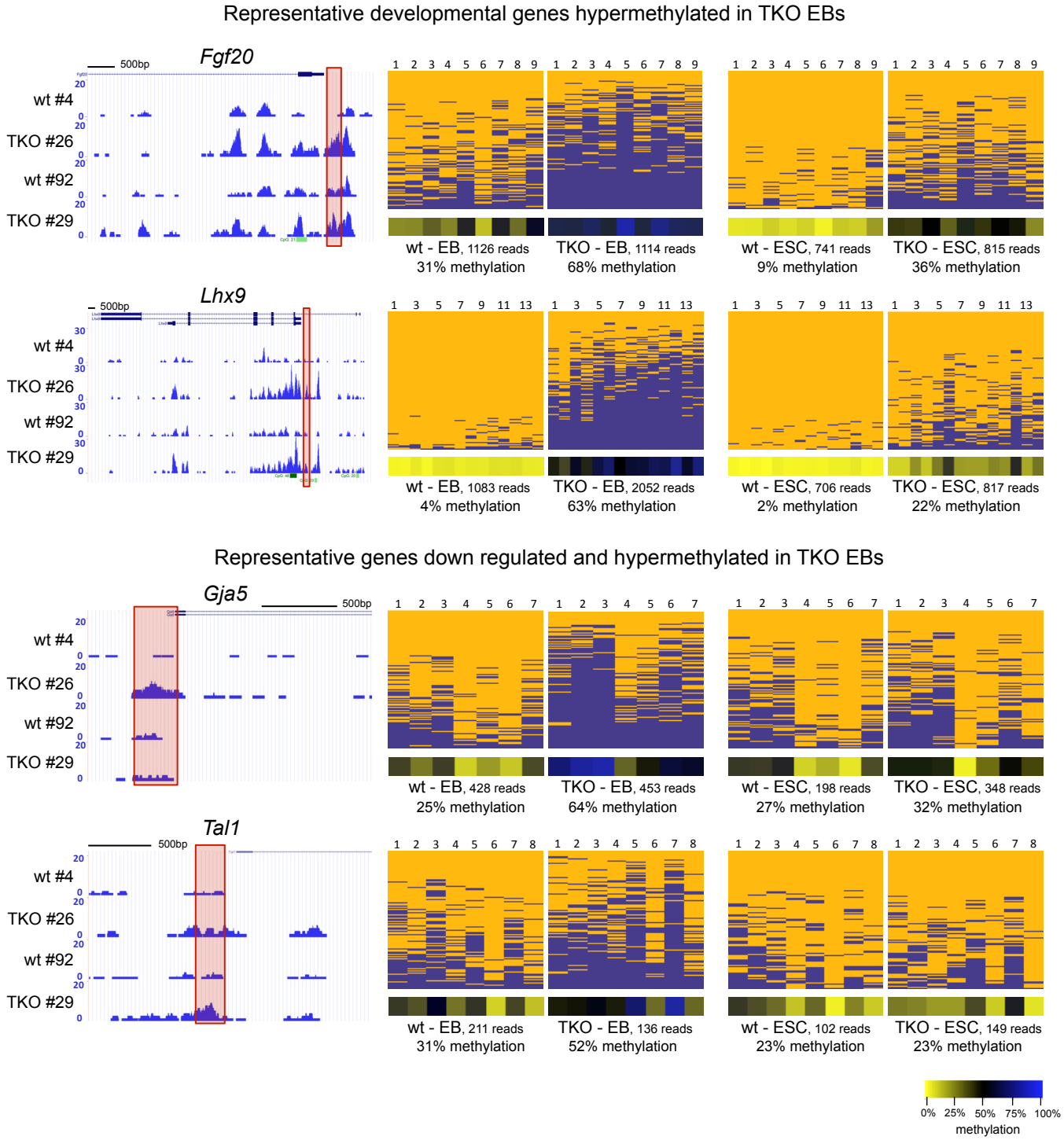

Figure S3: Additional examples of MeDIP-seq and 454 locus-specific BS-sequencing of hypermethylated genes in TKO EBs and ESCs (Related to Figure 4). *Left panels*: MeDIP-seq profiles of representative developmental genes hypermethylated in TKO EBs (*Fgf20* and *Lhx9*) and of two representative down regulated and promoter hypermethylated genes in TKO EBs (*Gja5* and *Tal1*). Enrichments are indicated as normalized read counts. Red boxes indicate the genomic region analyzed by gene specific 454-bisulfite sequencing in the right panels. *Right panels:* Validation of MeDIP-data by gene specific 454 bisulfite sequencing. Heatmaps show methylation status of the regions indicated on the left (see red box). Each row represents one sequence read. Individual blue boxes indicate methylated and yellow boxes indicate unmethylated CpG dinucleotides. Panels below heatmaps show the average methylation of each interrogated CpG for the analyzed DNA fragment samples according to the color bar shown on the bottom. Sequencing coverage (reads) are indicated. Number on top of the panels refer to CpG positions in the analyzed region.

Table S1: List of deregulated genes in Tet TKO EB when compared to WT EB in a microarray analysis (Related to Figure S3).

Supplemental Experimental Procedures

Generation of rescued TKO ESCs

For rescue experiments stable ESC lines expressing Tet1 cDNAs in TKO ES cells were generated by transfecting PiggyBac-Tet1-pgk-hygro or PiggyBac-empty-vector-pgk-hygro vectors (Costa et al., 2013) into TKO-R26-EGFP ESCs using X-fect mESC transfection reagent kit (Clonetech) following the manufacturer's protocols and selected with hygromycin for 12 days (130ug/mL). Expression of overexpressed Tet1 mRNA and 5hmC levels in the cells were confirmed by RTqPCR and mass spectrometry, respectively.

Tetraploid (4N) complementation

Tetraploid complementation assay was performed as described (Wernig et al., 2007). Briefly, 10-12 mESCs were injected into 4N blastocysts (generated by fusion of two-cell stage B6D2F1 x B6D2F1 embryos) following standard procedures. Twenty injected embryos were surgically transferred into the uterus of 2.5 d.p.c. pseudo-pregnant Swiss Webster female mice. Pregnant mice were sacrificed at day E9.5 and dissected to examine and genotype any developing embryo proper.

RTqPCRs

RNA was extracted from pre-plated ESCs or EBs using Qiagen RNAeasy kit. About 1.5µg of RNA was used to synthesize cDNA (Invitrogen Superscript III kit). Real time quantitative PCR was performed in an ABI 7900 cycler (Applied Biosystems) using primers for Pax6, Brachrury (Ito et al., 2010), Sox17 (Chew et al., 2011), Tet1, Tet2, Tet3 and Gapdh (Dawlaty et al., 2013). Gene expression was normalized to Gapdh.

Southern and western blots

Ten microgram of DNA was digested with restriction enzyme over night, analyzed on 0.9% agarose gel and transferred to nitrocellulose membrane. Transfer, hybridization of blots and probe sequences were as described before (Dawlaty et al., 2011). For Tet1/2/3 western blots, lysates preparation, gel and transfer were performed as described before(Dawlaty et al., 2011). Anti-Tet1 antibody (Millipore, 1:1000), anti-Tet2 (Abcam 1:250), anti Tet3 (Active Motif, 1:500) and mouse monoclonal anti-actin (Abcam, 1:40000) were used. Primary antibody incubation time was 3 hour at room temperature or over night at 4 C.

454 DNA bisulfite sequencing

Following primer pairs were used for PCR amplification of 200-300bp promoter elements of selected genes:

Emid2 (chr5:137,358,950-137,359,193) 14 CpGs: FOR 5'-GATTGATTTTGTTGTTTTAGGTTG-3' REV-5'-CATATATATCCCTAAAAATACCCCC-3'. *Fgf20* (chr8:41,372,337-41,372,646) 9 CpGs: For: 5'GGGGGTTGAGGTTTTAGTTTAGTA3', Rev: 5'AACATCATAATTATCCCAAAAACAAC3'. *Gja5* (chr3:96,836,062-96,836,350) 7 CpGs: For: 5'AGGATAGTTATTGGGATAGGGATAG3', Rev: 5'ACCCACTATTATTTACTCTTAAACCTATTA3'. *Lhx9* (chr1:140,740,157-140,740,371) 14 CpGs: For: 5'TTTTGATATTGTGGATTTATTGAAA3', Rev: 5'ACTTCTCCAACTTTCTAAACAAAAAC3'. *Mall* (chr2:127,555,594-127,555,793) 12 CpGs: For: 5'GGAGGTTATGTTGTTATTTTTGTTG3', Rev: 5'AATCCTATCCCCTCTCCTAACTAAC3'.

MeDIP-seq

MeDIP-seq was performed as described (Bocker et al., 2012) but significantly adapted and scaled down to smaller starting amounts according to the protocol by Taiwo and colleagues (Taiwo et al., 2012). Briefly, 2 µg of genomic DNA isolated from day 10 EBs was sonicated to 300bp fragments using a Covaris S220 ultrasonicator (Covaris). Fragmented DNA was end repaired, dA-tailed and ligated to

barcoded Illumina adapters using NEBNext sample preparation modules (New England Biolabs). After each enzymatic treatment DNA was purified using Agencourt AMPure XP beads (Beckman Coulter). One µg of adapter ligated DNA was used for pooled immunoprecipitations using a polyclonal 5mC-specific antibody (Active Motif, 39791). Sequencing libraries were then generated by pooling immunoprecipitated DNA from 8 samples. Libraries were amplified using Illumina Enrichment Primers. Paired-end 100 bp sequencing was performed on an Illumina HiSeq 2000 system.

Mapping of sequencing data and calculation of methylation levels

Reads were trimmed by removing stretches of bases having a quality score <30 at the ends and to a maximal length of 60 bp. The trimmed reads were mapped to the mouse genome assembly mm9 using the mapping program BOWTIE (Langmead et al., 2009; Dreszer et al., 2011), allowing up to 2 mismatches per read. After the mapping duplicate reads were removed using PICARD (http://picard.sourceforge.net) and the position-wise coverage of the genome by sequencing reads was determined and visualized as custom tracks in the UCSC genome browser (Dreszer et al., 2011). In order to quantitatively analyze sequencing reads, position-wise read counts were normalized according to the 5mC levels of each sample DNA, as determined by mass spectroscopy. Compartment-wise methylation for all genes defined by the RefSeq annotation (Pruitt et al., 2012) was computed by averaging over the normalized position-wise read coverage of all CpGs located in a particular compartment of a gene, i.e. promoter (+/– 1000 bp from the TSS) or gene body. Differences in methylation levels for each gene in different samples were calculated by subtracting the normalized average read counts in TKO samples from the respective WT values. If the differences was equal or bigger than 0.25 the region was considered hypermethylated in TKO. If the difference equal or smaller than -0.25, the region was considered as hypomethylated.

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